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(54) Title: PHOSPHOGLYCERATE KINASE IN THE TREATMENT OF DISEASE

(57) Abstract: The present invention provides a pharmaceutical composition for the inhibition of angiogenesis associated with disease in a vertebrate, said composition comprising phosphoglycerate kinase, or a fragment(s) or analogue thereof, together with a pharmaceutically acceptable carrier, adjuvant and/or diluent. The present invention also relates to the treatment of disease, especially cancer, in a vertebrate via the administration of a therapeutically effective amount of phosphoglycerate kinase (PGK), or fragment(s) or analogue thereof.

Phosphoglycerate Kinase in the Treatment of Disease

Technical Field

The present invention relates to the treatment of disease, especially cancer, in a vertebrate via the administration of a therapeutically effective amount of phosphoglycerate kinase (PGK), or a fragment(s) or analogue thereof.

Background Art

It has become clear in recent years that tumour expansion and metastasis is dependent on tumour neovascularization, or angiogenesis (*1, 2*). Tumours 1-2 mm in diameter can receive all nutrients and eliminate waste products by diffusion from existing blood vessels, however further expansion depends on the development of an adequate blood supply. The endothelial cells which line blood vessels are genetically stable, in contrast to tumour cells which are inherently unstable. Most tumour cells have a propensity for mutation and genetic diversity, and are therefore likely to produce drug resistant cells. The genetic stability of endothelial cells suggests that drugs that target proliferating endothelial cells in tumours will be less prone to resistance than the chemotherapeutic agents that target the tumour cells. One of the limitations of conventional disease therapies is that they are often ineffective in causing regression of tumours that have undergone distant or widespread metastasis. In contrast, one of the potential advantages of antiangiogenic therapy is that antiangiogenic agents can cause regression of metastatic lesions and also prevent dissemination.

Accordingly, there is a need for a compound effective in the inhibition of angiogenesis. One such inhibitor is angiostatin. Angiostatin is an internal fragment of the plasma zymogen, plasminogen. A rate limiting step in angiostatin formation is reduction of plasmin by plasmin reductase. In the present invention, the plasmin reductase has been shown to be the glycolytic enzyme, phosphoglycerate kinase (PGK). The findings outlined herein indicate that PGK not only functions in glycolysis, but is secreted by tumour cells and participates in the angiogenic process as a disulfide reductase.

Therefore, the present invention provides a useful therapeutic in the treatment of disease, in the form of phosphoglycerate kinase (PGK), or fragment(s) thereof.

Disclosure of the Invention

The present invention relates to the finding that the protein, phosphoglycerate kinase, or a fragment(s) or analogue thereof, is a useful therapeutic for the treatment of

disease, especially cancer. The results disclosed herein support the position that administration of phosphoglycerate kinase (PGK) or a fragment(s) or analogue thereof, facilitates formation of the tumour angiogenesis inhibitor, angiostatin, from endogenously produced plasmin, which will in turn inhibit angiogenesis associated with disease, as evidenced by slowing or stopping tumour growth. Further to this, the combination of PGK and at least one plasminogen activator, enhances angiostatin formation *in vivo,* and result in greater inhibition of tumour growth than that achievable through administration of PGK or a fragment(s) or analogue thereof, alone.

1. Therapeutic/Pharmaceutical Compositions for Inhibition of Angiogenesis

According to a first embodiment of the invention, there is provided a pharmaceutical composition for the inhibition of angiogenesis associated with disease in a vertebrate, said composition comprising phosphoglycerate kinase, or a fragment(s) or analogue thereof, together with a pharmaceutically acceptable carrier, adjuvant and/or diluent.

Typically, the phosphoglycerate kinase (PGK) is ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3. More typically, PGK is encoded by the isolated nucleic acid molecule defined in SEQ ID NO: 1, wherein the nucleic acid molecule typically corresponds to a DNA or RNA molecule.

Typically, the PGK protein or fragment(s) thereof is encoded by a wild type or mutant PGK gene.

Typically, the nucleic acid molecule also includes within its scope an analogue of the nucleic acid sequence defined above, wherein said analogue encodes a polypeptide having a biological activity which is functionally the same as the polypeptide (or fragment thereof) encoded by the nucleic acid molecule defined in SEQ ID NO: 1, wherein said nucleic acid sequence can be located and isolated using standard techniques in molecular biology, without undue trial and experimentation.

Typically, the nucleic acid molecule also includes within its scope an analogue which has at least 45% homology to the polynucleotide sequences so defined. More typically, the analogue of the nucleic acid molecule has at least 55% homology, still more typically the analogue has at least 60% homology, even more typically, the analogue has at least 75% homology, still more typically, the analogue has at least 85% homology, and yet still more typically, the analogue has at least 90% homology, and yet even still more

typically, the analogue has at least 95-99% homology to the nucleic acid molecule defined in SEQ ID NO: 1

The degree of homology between two nucleic acid sequences may be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1996, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), *Journal of Molecular Biology*, 48, 443-453). Using GAP with the following settings for DNA sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3.

Nucleic acid molecules may be aligned to each other using the Pileup alignment software, available as part of the GCG program package, using, for instance, the default settings of gap creation penalty of 5 and gap width penalty of 0.3.

Typically, the nucleic acid molecule also includes within its scope an analogue capable of hybridising to the nucleic acid molecule defined in SEQ ID NO: 1 under conditions of low stringency. More typically, low stringency hybridisation conditions correspond to hybridisation performed at 50°C in 6xSSC.

Suitable experimental conditions for determining whether a given nucleic acid molecule hybridises to a specified nucleic acid may involve presoaking of a filter containing a relevant sample of the nucleic acid to be examined in 5 x SSC for 10 min, and prehybridisation of the filter in a solution of 5 x SSC, 5 x Denhardt's solution, 0.5% SDS and 100 μg/ml of denatured sonicated salmon sperm DNA, followed by hybridisation in the same solution containing a concentration of 10 ng/ml of a ^{32}P-dCTP-labeled probe for 12 hours at approximately 45°C, in accordance with the hybridisation methods as described in Sambrook *et al.* (1989; Molecular Cloning, A Laboratory Manual, 2nd edition, Cold Spring Harbour, New York).

The filter is then washed twice for 30 minutes in 2 x SSC, 0.5% SDS at least 55° C (low stringency), at least 60°C (medium stringency), at least 65°C (medium/high stringency), at least 70°C (high stringency), or at least 75°C (very high stringency). Hybridisation may be detected by exposure of the filter to an x-ray film.

Further, there are numerous conditions and factors, well known to those skilled in the art, which may be employed to alter the stringency of hybridisation. For instance, the length and nature (DNA, RNA, base composition) of the nucleic acid to be hybridised to a specified nucleic acid; concentration of salts and other components, such as the

presence or absence of formamide, dextran sulfate, polyethylene glycol etc; and altering the temperature of the hybridisation and/or washing steps.

Further, it is also possible to theoretically predict whether or not two given nucleic acid sequences will hybridise under certain specified conditions. Accordingly, as an alternative to the empirical method described above, the determination as to whether an analogous nucleic acid sequence will hybridise to the nucleic acid molecule defined in SEQ ID NO:1, can be based on a theoretical calculation of the T_m (melting temperature) at which two heterologous nucleic acid sequences with known sequences will hybridise under specified conditions, such as salt concentration and temperature.

In determining the melting temperature for heterologous nucleic acid sequences ($T_{m(hetero)}$) it is necessary first to determine the melting temperature ($T_{m(homo)}$) for homologous nucleic acid sequence. The melting temperature ($T_{m(homo)}$) between two fully complementary nucleic acid strands (homoduplex formation) may be determined in accordance with the following formula, as outlined in Current Protocols in Molecular Biology, John Wiley and Sons, 1995, as:

$$T_{m(homo)} = 81.5°C + 16.6(\log M) + 0.41(\%GC) - 0.61\ (\%\ form) - 500/L$$

M = denotes the molarity of monovalent cations,

%GC = % guanine (G) and cytosine (C) of total number of bases in the sequence,

% form = % formamide in the hybridisation buffer, and

L = the length of the nucleic acid sequence.

T_m determined by the above formula is the T_m of a homoduplex formation ($T_{m(homo)}$) between two fully complementary nucleic acid sequences. In order to adapt the T_m value to that of two heterologous nucleic acid sequences, it is assumed that a 1% difference in nucleotide sequence between two heterologous sequences equals a 1°C decrease in T_m. Therefore, the $T_{m(hetero)}$ for the heteroduplex formation is obtained through subtracting the homology % difference between the analogous sequence in question and the nucleotide probe described above from the $T_{m(homo)}$.

Typically the nucleic acid molecule defined in SEQ ID NO:1 also includes within its scope a nucleic acid molecule which is an oligonucleotide fragment thereof. Typically, the oligonucleotide fragment is between about 15 to about 750 nucleotides in length. More typically, the oligonucleotide fragment is between about 15 to about 600

nucleotides in length. Even more typically, the oligonucleotide fragment is between about 15 to about 150 nucleotides in length. Even more typically still, the oligonucleotide fragment is between about 15 to about 90 nucleotides in length. Yet still more typically, the oligonucleotide fragment is between about 15 to about 75 nucleotides in length.

Typically, PGK corresponds to the polypeptide comprising the amino acid sequence defined in SEQ ID NO: 2.

Typically, the polypeptide also includes within its scope an analogue of the polypeptide comprising the amino acid sequence defined in SEQ ID NO:2, wherein said analogue comprises a polypeptide having a biological activity which is functionally the same as the polypeptide (or fragment thereof) comprising the amino acid sequence defined in SEQ ID NO: 2.

Typically, the polypeptide comprising the amino acid sequence defined in SEQ ID NO: 2 includes within its scope a peptide fragment of the polypeptide. Typically, the peptide fragment is between about 5 to about 250 contiguous amino acids. More typically, the peptide fragment is between about 5 to about 200 contiguous amino acids. Still more typically, the peptide fragment is between about 5 to about 150 contiguous amino acids. Yet still more typically, the peptide fragment is between about 5 to about 100 contiguous amino acids. More typically, the peptide fragment is between about 5 to about 50 contiguous amino acids. Even more typically, between about 5 to about 35 contiguous amino acids. Still even more typically, between about 5 to about 30 contiguous amino acids. Yet more typically, between about 5 to about 25 contiguous amino acids. Yet still more typically, between about 5 to about 20 contiguous amino acids.

Typically, the peptide fragment is the C-terminal domain of PGK.

Typically, the polypeptide also includes within its scope a homologous polypeptide of the polypeptide comprising the amino acid sequence defined in SEQ ID NO: 2, which has at least 35% homology to the polypeptide sequences so defined. More typically, the homologue of the polypeptide sequences has at least 45% homology, still more typically the homologue has at least 65% homology, even more typically, the homologue has at least 75% homology, still more typically, the homologue has at least 85% homology, and yet still more typically, the homologue has at least 90% homology, and yet even still more typically, the homologue has at least 95-99% homology to the polypeptide sequences so defined.

As applied to polypeptides, the degree of homology between two polypeptide sequences when optimally aligned, may be determined through the use of computer alignment programs known in the art such as, for example: BLAZE (Intelligenetics) GAP, BESTFIT, ALIGN, using default gap weights. One specific example is the GAP program as provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1996, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453), using the following settings for sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3.

Typically, the PGK polypeptide or fragment thereof present in the pharmaceutical composition may also exist in a form selected from the group consisting of: PGK/chelate, PGK/drug, PGK/prodrug, PGK/toxin and PGK/detector group and PGK/imaging marker. More typically, the chelate may be selected from the group consisting of: gadolinium, ^{90}Y, ^{131}I and ^{188}Re. More typically, the drug may be a cytotoxic drug. Even more typically, the cytotoxic drug may be selected from the group consisting of: adriamycin, melphalan, cisplatin, taxol, fluorouricil, cyclophosphamide and others known to those of skill in the art, such as those included in "The Chemotherapy Source Book", M.C.Perry Williams and Wilkins, 2nd Ed, 1996), the entire contents of which are incorporated herein by reference. More typically, the toxin may be selected from the group consisting of: ricin, abrin, *Diptheria* toxin and *Pseudomonas* endotoxin (PE 40).

Typically, the PGK polypeptide or fragment thereof present in the pharmaceutical composition may also may be linked to detector groups. Typically, the detector group may be a chemical group, such as biotin, streptavidin or dioxigenin. Alternatively, the detector group is a radionuclide, such as ^{3}H, ^{14}C, ^{32}P, ^{33}P, ^{35}S, ^{125}I, ^{131}I, ^{123}I, ^{111}In, ^{105}Rh, ^{153}Sm, ^{67}Cu, ^{67}Ga, ^{166}Ho, ^{177}Lu, ^{186}Re, ^{188}Re, and ^{99m}Tc.

Typically, the imaging marker includes substances which can be detected by a gamma scanner or hand held gamma probe, and substances which can be detected by nuclear magnetic resonance imaging using a nuclear magnetic resonance spectrometer. More typically, the imaging marker which may be detected using a gamma scanner include imaging markers selected from the group consisting of ^{125}I, ^{131}I, ^{123}I, ^{111}In, ^{105}Rh, ^{153}Sm, ^{67}Cu, ^{67}Ga, ^{166}Ho, ^{177}Lu, ^{186}Re, ^{188}Re, and ^{99m}Tc. Typically, the imaging marker which can be detected using a nuclear magnetic resonance spectrometer, is gadolinium.

Typically, the pharmaceutical composition in accordance with the first embodiment of the invention may also include cytokines, such as: G-CSF, GM-CSF, and the interleukins.

Typically, the vertebrate is selected from the group consisting of human, non-human primate, murine, bovine, ovine, equine, caprine, leporine, avian, feline and canine. More typically, the vertebrate is human, non-human primate or murine. Even more typically, the vertebrate is human.

Typically, the nucleic acid molecule as defined in SEQ ID NO: 1 or fragment(s) thereof is present in a vector. Typically, the vector is a shuttle or expression vector. More typically, the vector is selected from the group consisting of: viral, plasmid, bacteriophage, phagemid, cosmid, bacterial artificial chromosome, and yeast artificial chromosome.

Typically, the vector is a plasmid and may be selected from the group consisting of: pBR322, M13mp18, pUC18 and pUC19. Typically, the vector is a bacteriophage and may be selected from λgt10 and λgt11 or phage display vectors.

Typically, the vector includes expression control sequences, such as an origin of replication, a promoter, an enhancer, and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Still more typically, the vector may include selection markers to permit detection of those cells transformed with the desired polynucleotide sequences.

Typically, the vector may include heterologous coding sequence or sequences to permit the expression of a fusion protein comprising the polypeptide encoded by the nucleic acid molecule as defined in SEQ ID NO: 1, or the polypeptide as defined in SEQ ID NO: 2.

Typically, the vector is transformed into a host cell. More typically, the host cells are procaryotic or eucaryotic in nature. Typically, the procaryotic host cells include bacteria, and examples of such bacteria include: *E. coli, Bacillus, Streptomyces, Pseudomonas, Salmonella,* and *Serratia.* Typically, the eucaryotic host cells may be selected from the group consisting of: yeast, fungi, plant, insect cells and mammalian cells, either *in vivo* or in tissue culture. Examples of mammalian cells include: CHO cell lines, COS cell lines, HeLa cells, L cells, murine 3T3 cells, c6 glioma cells or myeloma cell lines.

According to a second embodiment of the invention, there is provided a process for preparing a pharmaceutical composition as defined in the first embodiment of the

invention, wherein said process comprises homogeneously mixing a phosphoglycerate kinase, or a fragment(s) or analogue thereof, with a pharmaceutically acceptable carrier, adjuvant and/or diluent.

2. Treatment of Disease using PGK

According to a third embodiment of the invention, there is provided a method for the treatment of disease in a vertebrate in need of said treatment, wherein said method comprises administering to said vertebrate, a therapeutically effective amount of phosphoglycerate kinase, or a fragment(s) or analogue thereof.

According to a fourth embodiment of the invention, there is provided phosphoglycerate kinase, or a fragment(s) or analogue thereof when used in the treatment of disease in a vertebrate in need of said treatment.

According to a fifth embodiment of the invention, there is provided use of phosphoglycerate kinase, or a fragment(s) or analogue thereof, for the preparation of a medicament for the treatment of disease in a vertebrate in need of said treatment.

According to a sixth embodiment of the invention, there is provided a method for the treatment of disease in a vertebrate in need of said treatment, wherein said method comprises administering to said vertebrate, a therapeutically effective amount of the pharmaceutical composition as defined in the first embodiment of the invention.

According to a seventh embodiment of the invention, there is provided a pharmaceutical composition as defined in the first embodiment of the invention, when used in the treatment of disease in a vertebrate in need of said treatment.

According to an eighth embodiment of the invention, there is provided use of the pharmaceutical composition as defined in the first embodiment of the invention for the preparation of a medicament for the treatment of disease in a vertebrate in need of said treatment.

3. PGK/Plasminogen Activator Compositions for Inhibition of Angiogenesis

According to a ninth embodiment of the invention, there is provided a pharmaceutical composition for inhibition of angiogenesis associated with disease in a vertebrate, said composition comprising phosphoglycerate kinase, or a fragment(s) or analogue thereof, and at least one plasminogen activator.

Typically, the composition in accordance with the ninth embodiment of the invention is synergistic in nature. That is, the effect of the composition in accordance with the ninth embodiment of the invention on the inhibition of angiogenesis associated

with disease in a vertebrate is greater than the individual effects of PGK or a fragment(s) thereof, and at least one plasminogen activator, when considered separately.

Typically, the plasminogen activator is selected from the group consisting of: streptokinase, tissue plasminogen activator, staphylokinase or urokinase plasminogen activator.

Typically, the composition in accordance with the ninth embodiment of the invention also includes a pharmaceutically acceptable carrier, adjuvant and/or diluent.

According to a tenth embodiment of the invention, there is provided a process for preparing a pharmaceutical composition according to the ninth embodiment of the invention, wherein said process comprises mixing a phosphoglycerate kinase, or a fragment(s) thereof, and at least one plasminogen activator with a pharmaceutically acceptable carrier, adjuvant and/or diluent.

4. Treatment of Disease using PGK/Plasminogen Activator

According to an eleventh embodiment of the invention, there is provided a method for the treatment of disease in a vertebrate in need of said treatment, wherein said method comprises administering to said vertebrate a therapeutically effective amount of the composition as defined in the ninth embodiment of the invention.

According to a twelfth embodiment of the invention, there is provided the composition as defined in the ninth embodiment of the invention, when used in the treatment of disease in a vertebrate in need of said treatment.

According to a thirteenth embodiment of the invention, there is provided use of the composition as defined in the ninth embodiment of the invention, for the preparation of a medicament for the treatment of disease in a vertebrate in need of said treatment.

According to a fourteenth embodiment of the invention, there is provided a method for the treatment of disease in a vertebrate in need of said treatment, wherein said method comprises administering to said vertebrate, a therapeutically effective amount of phosphoglycerate kinase, or a fragment(s) or analogue thereof, and at least one plasminogen activator.

According to a fifteenth embodiment of the invention, there is provided phosphoglycerate kinase, or a fragment(s) or analogue thereof, and at least one plasminogen activator, when used in the treatment of disease in a vertebrate in need of said treatment.

According to a sixteenth embodiment of the invention, there is provided use of phosphoglycerate kinase, or a fragment(s) or analogue thereof, and at least one plasminogen activator, for the preparation of a medicament for the treatment of disease in a vertebrate in need of said treatment.

Typically, the plasminogen activator is selected from the group consisting of: streptokinase, tissue plasminogen activator, staphylokinase or urokinase plasminogen activator.

In a manner similar to that described in relation to the synergistic PGK/plasminogen activator composition in accordance with the ninth embodiment of the invention, the method, compounds or use of the fourteenth, fifteenth or sixteenth embodiments of the invention respectively, also reflect a synergistic effect upon angiogenesis and/or inhibition of disease, in terms of the response produced when PGK and at least one plasminogen activator are administered simultaneously, as opposed to PGK, or PGK and at least one plasminogen activator being administered separately.

Typically, with respect to any one of the third through to eighth or eleventh through to sixteenth embodiments of the invention, the treatment of disease through the administration of a therapeutically effective amount of PGK, or PGK and at least one plasminogen activator, is undertaken in conjunction with other treatments of disease. For example, these other treatments may include: surgery, radiation treatment, or chemotherapy.

Typically, for the purposes of any one of the third through to eighth or eleventh through to sixteenth embodiments of the invention, one skilled in the art would be able, by routine experimentation, to determine what an effective, non-toxic amount of PGK, or PGK and a plasminogen activator would be for the purpose of treating the disease.

5. Diagnosis of Disease

According to a seventeenth embodiment of the invention, there is provided a method for screening for a disease in a vertebrate comprising:

(a) contacting a sample from a vertebrate with an antibody (or fragment thereof) raised against a PGK polypeptide (or fragment or analogue thereof), and

(b) detecting the presence of the antibody (or fragment thereof) bound to the PGK polypeptide.

Typically, the sample within which the method of screening is performed is a plasma sample.

Typically, plasma PGK levels correlate with angiogenesis. More typically, a healthy vertebrate is expected to have little or no plasma PGK.

Typically, as described throughout the specification, the antibody may be a whole antibody, or an antibody fragment, or other immunologically active fragments thereof, such as complementarity determining regions. More typically, the antibody fragment has functional antigen-binding domains, that is, heavy and light chain variable domains. Even more typically, the antibody fragment may exist in a form selected from the group consisting of: Fv, F_{ab}, $F(ab)_2$, scFv (single chain Fv), dAb (single domain antibody), bi-specific antibodies, diabodies and triabodies.

Typically, the antibody (or fragment thereof) is a polyclonal or monoclonal antibody. More typically, the antibody (or fragment thereof) is a monoclonal antibody. Even more typically, the monoclonal antibody is generated using molecular genetic, hybridoma or EBV (Epstein-Barr virus) transformation technology.

According to an eighteenth embodiment of the invention, there is provided a diagnostic kit for the detection of disease in a vertebrate, said kit comprising at least an antibody (or fragment thereof) raised against PGK (or fragment thereof), together with a diagnostically acceptable carrier and/or diluent.

Typically, the kit may comprise the following containers:

(a) a first container containing at least the antibody (or fragment thereof) raised against PGK (or fragment thereof), and;

(b) a second container containing a conjugate comprising a binding partner of the antibody (or fragment thereof), together with a detectable label.

More typically, the kit may further comprise one or more other containers, containing other components, such as wash reagents, and other reagents capable of detecting the presence of bound antibodies. Even more typically, the detection reagents may include: labelled (secondary) antibodies, or where the antibody (or fragment thereof) raised against PGK (or fragment thereof), the compartments may comprise antibody binding reagents capable of reacting with the labelled antibody (or fragment thereof) of the present invention.

Typically, for the purposes of any one of the third through to eighth or eleventh through to eighteenth embodiments of the invention, the disease involves angiogenesis, and is even more typically, cancer.

6. Increasing Circulat ry PGK

According to an nineteenth embodiment of the invention, there is provided a method for increasing circulatory phosphoglycerate kinase (PGK) levels in a vertebrate in need of said increase, wherein said method comprises:

(a) generating anti anti-PGK antibodies and/or fragments thereof; and

(b) administering a therapeutically effective amount of said anti anti-PGK antibodies and/or fragments thereof to said vertebrate.

According to a twentieth embodiment of the invention, there is provided anti anti-PGK antibodies and/or fragments thereof, when used in increasing circulatory PGK levels in a vertebrate in need of said increase.

According to a twenty-first embodiment of the invention, there is provided use of anti anti-PGK antibodies and/or fragments thereof, in the preparation of a medicament for increasing circulatory PGK levels in a vertebrate in need of said increase.

Typically, the method, antibodies or use in accordance with the nineteenth twentieth or twenty-first embodiments of the invention reduce the removal of circulating PGK by anti-PGK antibodies, thereby effectively elevating circulating PGK levels, and thus increasing the level of the angiogenesis inhibitor -angiostatin.

According to a twenty-second embodiment of the invention, there is provided a method for inhibiting the action of excess endogenous phosphoglycerate kinase in a vertebrate in need of said inhibition, wherein said method comprises passively immunising said vertebrate with anti-PGK antibodies and/or fragments thereof.

According to a twenty-third embodiment of the invention, there is provided anti-PGK antibodies and/or fragments thereof when used in inhibiting the action of excess endogenous phosphoglycerate kinase in a vertebrate in need of said inhibition.

According to a twenty-fourth embodiment of the invention, there is provided use of anti-PGK antibodies and/or fragments thereof in the preparation of a medicament for inhibiting the action of excess endogenous phosphoglycerate kinase in a vertebrate in need of said inhibition.

7. Gene Therapy

According to a twenty-fifth embodiment of the invention, there is provided a method of gene therapy for the inhibition of angiogenesis associated with disease in a vertebrate, wherein said method comprises:

(a) inserting a nucleic acid molecule encoding for a phosphoglycerate kinase (PGK), or fragment(s) or analogue thereof, or a vector comprising a nucleic acid molecule encoding for a phosphoglycerate kinase or a fragment(s) or analogue thereof, into a host cell;

(b) expressing the nucleic acid molecule in the transformed cell.

Typically, the nucleic acid molecule or vector is inserted using methods selected from the group consisting of: microinjection, $CaPO_4$ precipitation, electroporation, lipofection/liposome fusion, particle bombardment and coupling the nucleic acid to chemically modified proteins.

Typically the nucleic acid molecule or vector is inserted into the nucleus of a host cell.

Typically, an expression vector containing the nucleic acid molecule is inserted into cells, the cells are grown *in vitro* and then infused in large numbers into patients. More typically, expression vectors derived from viruses such as retroviruses, vaccinia virus, adenovirus, adeno-associated virus, herpes viruses, several RNA viruses, or bovine papilloma virus, may be used for delivery of the nucleic acid into the targeted cell population. More typically, the targeted cell population comprises tumour cells.

Definitions

The term "angiogenesis" means the generation of new blood vessels from existing blood vessels in a tissue or organ.

The term "nucleic acid" encompasses deoxyribonucleotide (DNA) and/or ribonucleotide (RNA) nucleic acid, either in the single or double-stranded form, and includes within its scope all known analogues of natural nucleotides.

The term "polynucleotide" encompasses deoxyribopolynucleotide and/or ribopolynucleotide, either in the single or double-stranded form, and includes within its scope all known analogues of natural nucleotides. Also, it includes within its scope the relevant sequence as specified, together with the sequence complementary thereto.

As used herein the term "polypeptide" means a polymer made up of amino acids linked together by peptide bonds.

The term "antibody" means an immunoglobulin molecule able to bind to a specific epitope on an antigen. Antibodies can be comprised of a polyclonal mixture, or may be monoclonal in nature. Further, antibodies can be entire immunoglobulins derived from natural sources, or from recombinant sources. The antibodies of the present

invention may exist in a variety of forms, including for example as a whole antibody, or as an antibody fragment, or another immunologically active fragment thereof, such as complementarity determining regions. Similarly, the antibody may exist as an antibody fragment having functional antigen-binding domains, that is, heavy and light chain variable domains. Also, the antibody fragment may exist in a form selected from the group consisting of: Fv, F_{ab}, $F(ab)_2$, scFv (single chain Fv), dAb (single domain antibody), bi-specific antibodies, diabodies and triabodies.

A "therapeutically effective amount", as used herein, includes within its meaning a non-toxic but sufficient amount a compound or composition of the invention to provide the desired therapeutic effect. The exact amount required will vary from subject to subject depending on factors such as the species being treated, the age and general condition of the subject, the severity of the condition being treated, the particular agent being administered and the mode of administration and so forth. Thus, it is not possible to specify an exact "effective amount". However, for any given case, an appropriate "effective amount" may be determined by one of ordinary skill in the art using only routine experimentation. Typically, "therapeutically effective amount" refers to an amount sufficient to result in one or more or the following: reduction in the size of the disease, inhibition of disease metastasis, inhibition of disease growth, stop disease growth, relieve disease imposed discomfort, or prolong life of the vertebrate having the disease.

The term "isolated" means that the material in question has been removed from its host, and associated impurities reduced or eliminated. Essentially, it means an object species is the predominant species present (ie., on a molar basis it is more abundant than any other individual species in the composition), and preferably a substantially purified fraction is a composition wherein the object species comprises at least about 30 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80 to 90 percent of all macromolecular species present in the composition. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

"Conservative amino acid substitutions" refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids

having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Typically, conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

The term "fragment" of a compound is a compound having qualitative biological activity in common with for example, a full-length polypeptide.

The term "analogue" as used herein with reference to a nucleic acid sequence means a sequence which is a derivative of the nucleic acid sequences of the invention, which derivative comprises addition, deletion, substitution of one or more bases and wherein the encoded polypeptide retains substantially the same function as the PGK polypeptide encoded by the nucleic acid sequence defined above. Similarly, the term "analogue" as used herein with reference to a polypeptide means a polypeptide which is a derivative of the polypeptide of the invention, which derivative comprises addition, deletion, substitution of one or more amino acids, such that the polypeptide retains substantially the same function as the PGK polypeptide identified above.

The term "wild-type", in terms of a gene or a gene product, refers to that gene or a gene product which is characteristic of most of the members of a species occurring naturally, and is thus arbitrarily designated the "normal" or "wild-type" form of the gene or gene product.

The term "mutant", in terms of a gene or gene product, refers a change in the gene or gene product when compared to the wild-type gene or gene product.

The term "expression cassette" refers to a nucleic acid construct comprising a number of nucleic acid elements (promoters, enhancers, the nucleic acid to be transcribed, etc) which permit the transcription of the particular nucleic acid in a host cell. The expression construct can be incorporated into a vector, host chromosome etc.

The term "promoter" refers to nucleic acid sequences that influence and/or promote initiation of transcription.

The term "operably linked" refers to the situation wherein for example, a nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, a promoter operably linked to a heterologous DNA,

which encodes a protein, promotes the production of functional mRNA corresponding to the heterologous DNA

The term "synergistic composition" relates to any property of the compositions of the present invention, which is advantageous, compared with the combined corresponding individual properties of phosphoglycerate kinase and at least one plasminogen activator together forming the active ingredients of the synergistic composition.

The term "simultaneous" is used to mean administration of PGK and a plasminogen activator within a period wherein the PGK and at least one plasminogen activator remain active within the vertebrate, typically such a period is at least 24 hours. That is, it is not essential that PGK and at least one plasminogen activator be administered together in order to provide a synergistic therapeutic response.

As used herein "gene transfer" means the process of introducing a foreign nucleic acid molecule into a cell. Gene transfer is commonly performed to enable the expression of a particular product encoded by the gene. The product may include a protein, polypeptide, anti-sense DNA or RNA, or enzymatically active RNA. Gene transfer can be performed in cultured cells or by direct administration into animals. Generally gene transfer involves the process of nucleic acid contact with a target cell by non-specific or receptor mediated interactions, uptake of nucleic acid into the cell through the membrane or by endocytosis, and release of nucleic acid into the cytoplasm from the plasma membrane or endosome. Expression may require, in addition, movement of the nucleic acid into the nucleus of the cell and binding to appropriate nuclear factors for transcription.

As used herein "gene therapy" is a form of gene transfer and is included within the definition of gene transfer as used herein and specifically refers to gene transfer to express a therapeutic product from a cell *in vivo* or *in vitro*. Gene transfer can be performed *ex vivo* on cells which are then transplanted into a patient, or can be performed by direct administration of the nucleic acid or nucleic acid-protein complex into the patient, or can be performed by transfer of modified cells into a patient.

As used herein the term "treatment", refers to any and all uses which remedy a disease state or symptoms, or otherwise prevent, hinder, retard, or reverse the progression of disease or other undesirable symptoms in any way whatsoever.

In the context of this specification, the term "comprising" means "including principally, but not necessarily solely". Furthermore, variations of the word

"comprising", such as "comprise" and "comprises", have correspondingly varied meanings.

Abbreviations

The following abbreviations are used throughout the specification: cm: conditioned medium; EACA: ε-amino-caproic acid; EDTA: ethylenediamine tetraacetic acid; GSH: reduced glutathione; MPB: 3-(N-maleimidylpropionyl)biocytin; PBS: phosphate buffered saline; rhPGK: recombinant human phosphoglycerate kinase; SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Brief Description of the Drawings

Figure 1: Assay and purification of plasmin reductase. **a,** Samples were incubated with plasmin (2 μg per ml) in Hepes-buffered saline for 30 min at 37°C. The control reaction was plasmin incubated in Hepes-buffered saline. The angiostatin fragments formed were labeled with MPB and detected by ELISA. For controls, HT1080 conditioned medium (HT1080cm) was incubated without plasmin and the MPB labeling of a HT1080cm/plasmin reaction was omitted on one occasion (open bars). The bars are the mean and SE of triplicate determinations. **b,** Plasmin (2 μg per ml) was incubated in dilutions of HT1080cm in Hepes-buffered saline for 30 minutes at 37°C and the angiostatin fragments labeled with MPB and detected by ELISA. The data has been corrected for the background absorbance from incubation of plasmin in Hepes buffer alone (see part a). The dotted line represents the linear least squares fit to the three right hand data points ($r^2 = 0.99$). The data points and errors are the mean and SE of triplicate determinations. **c,** Conditioned medium from HT1080 cells was applied to Cibachron Blue-Sepharose and the bound proteins were eluted with a linear NaCl gradient. The horizontal bar indicates the fractions that were pooled. **d,** The DTT eluate from the activated thiopropyl-Sepharose column was gel filtered on Sephacryl S-200 HR. The horizontal bar indicates the fractions that were pooled. The inset is the pooled plasmin reductase resolved on 4-15% SDS-PAGE and silver stained.

Figure 2: Amino acid sequence of human phosphoglycerate kinase. There was a 100% sequence identity between the HT1080 plasmin reductase (residues in italics) and phosphoglycerate kinase.

Figure 3: Recombinant human PGK has plasmin reductase activity. Human PGK cDNA was cloned by RT-PCR from HT1080 RNA. Human recombinant PGK protein was produced in *E. coli* and purified. **a,** HT1080 PGK and rhPGK (2 μg) were resolved on 4-

15% SDS-PAGE and stained with Coomassie Brilliant Blue. **b,** Plasmin (20 μg) was incubated with rhPGK (40 μg) in 0.5 mls of Hepes-buffered saline for 30 minutes at 37°C and the angiostatin fragments labeled with MPB (see Fig. 1a). The plasmin products were collected on lysine-Sepharose beads and either separated on 4-15% SDS-PAGE and silver stained (left hand panel), or separated on 10% SDS-PAGE and blotted with streptavidin-peroxidase to detect the MPB label (right hand panel). The arrows indicate the three angiostatin fragments. **c,** Plasmin (2 μg per ml) was incubated with either HT1080 PGK, rhPGK, rabbit PGK or yeast PGK (5 μg per ml) in Hepes-buffered saline for 30 minutes at 37°C and the angiostatin fragments labeled with MPB and detected by ELISA. The bars and errors are the mean and SE of triplicate determinations.

Figure 4: The C-terminal domain of PGK has plasmin reductase activity. The first 189 codons of hPGK cDNA were deleted and a C-terminal peptide of 23 kDa (amino acids 190 to 417) was expressed and purified as for full length PGK. **a,** rhPGK-C terminal fragment (2 μg) was resolved on 4-15% SDS-PAGE and stained with Coomassie Brilliant Blue. **c,** Plasmin (20 nM) was incubated with either rhPGK or rhPGK-C terminal fragment (0.5, 5 or 50 nM) in Hepes-buffered saline for 30 minutes at 37°C and the angiostatin fragments labeled with MPB and detected by ELISA (see Fig. 1a). The bars and errors are the mean and SE of triplicate determinations.

Figure 5: PGK is secreted by cultured carcinoma cells. Serum-free conditioned medium from HT1080 cells and several human pancreatic, breast and colon carcinoma cells was collected for 24 hours and the weight of PGK secreted from one million cells determined. The bars and errors represent the mean and SD of triplicate determinations.

Figure 6: PGK is secreted by tumours and administration of PGK to tumour-bearing mice caused an increase in plasma levels of angiostatin containing free thiols. HT1080 tumours were established subcutaneously in the proximal midline dorsum of SCID mice. Blood was collected from the retroorbital plexus of mice without (n = 4) and with (n = 4) tumours and one hour later they were administered 10 mg per kg of rhPGK in PBS via a 0.2 ml intraperitoneal injection. The mice were bled again after two hours. Blood was collected into PBS containing EDTA (20 mM), aprotinin (5 μM) and MPB (200 μM), and cysteine (400 μM) was added after 60 minutes to quench unreacted MPB. Plasma was separated and assayed for PGK (part **a**) or MPB-labeled angiostatin (part **b**). The mean tumour weight was 5.7 ± 1.0 g. The mean weight of the control and tumour bearing mice

was 23 ± 3 g and 22 ± 2 g, respectively. The bars and errors are the mean and SE of the four mice plasmas.

Figure 7: Administration of PGK to tumour-bearing mice resulted in decrease in tumour vascularity and rate of tumour growth. HT1080 (part **a**) or AsPC-1 (part **b**) tumours were established in the proximal midline dorsum of SCID mice. When the tumours were ~0.1 cm^3 in volume the mice were randomised into two groups (n = 4) and treated with either vehicle (PBS) or 5 mg per kg per day of rhPGK via 0.2 ml intraperitoneal injections. The arrow indicates the start of treatment. The data points and errors represent the mean and SE of the tumour volumes. **c**, The mice described in part b were sacrificed at day 16 of treatment and the AsPC-1 tumours were excised. **d**, Histological sections of the AsPC-1 tumours shown in part c were analyzed for vascularity by immunostaining for CD31.

Figure 8: Plasma of cancer patients contain elevated levels of PGK. Blood was collected by venipuncture into EDTA and plasma was prepared by centrifugation. Plasma was collected from 5 healthy subjects and from 8 patients with non-identified metastatic breast or colon cancer and assayed for PGK by ELISA. The mean absorbance and SE of the healthy subject plasma is shown. The cancer patient plasmas (#) are shown individually. The bars and errors are the mean and SE of triplicate determinations.

Sequence Listing

SEQ ID NO: 1: Nucleic acid sequence (DNA) encoding PGK

SEQ ID NO: 2: Deduced amino acid sequence of PGK

Best Mode of Performing the Invention

Angiogenesis is normally observed in wound healing, foetal and embryonal development and formation of the corpus luteum, endometrium and placenta. Angiogenesis begins with the erosion of the basement membrane by enzymes released by endothelial cells and leucocytes. The endothelial cells, which line the lumen of blood vessels, then protrude through the basement membrane. Angiogenic stimulants induce the endothelial cells to migrate through the eroded basement membrane. These migrating cells form a "sprout" off the parent blood vessel, and the endothelial sprouts merge with each other to form capillary loops, thereby creating a new blood vessel.

Persistent, unregulated angiogenesis occurs in a multiplicity of disease states, and supports the pathological damage seen in these conditions. The diverse pathological disease states in which unregulated angiogenesis is present have been grouped together as angiogenic dependent or angiogenic associated diseases.

As described above, tumour expansion is dependent on angiogenesis. Angiogenesis is balanced by several protein activators and inhibitors (*1*). One such inhibitor is angiostatin. The angiostatin produced by the primary tumour inhibits the neovascularization and growth of its remote metastases.

Angiostatin is an internal fragment of the plasma zymogen, plasminogen. Plasminogen contains five consecutive kringle domains followed by a serine proteinase module. Urokinase- or tissue-plasminogen activator convert plasminogen to plasmin by hydrolysis of the Arg^{561}-Val^{562} peptide bond in the serine proteinase module. Plasmin is processed in the conditioned medium of transformed cells and vascular smooth muscle cells producing angiostatin fragments consisting of kringle domains 1 to 4 and parts of kringle 5 (5). Plasmin proteolysis occurs in two stages. Firstly, the Cys^{461}-Cys^{540} and Cys^{511}-Cys^{535} disulfide bonds in kringle 5 of plasmin are reduced by a plasmin reductase. The plasmin reductase requires a small cofactor for activity, and physiologically relevant concentrations of reduced glutathione or cysteine fulfil this role. Secondly, reduction of the kringle 5 disulfide bonds triggers cleavage at Arg^{529}-Lys^{530} in kringle 5, and also at two other positions C-terminal of Cys^{461}, by a serine proteinase.

The rate limiting step in angiostatin formation by carcinoma cells is reduction of plasmin by plasmin reductase. In one form of the present invention, plasmin reductase was isolated from HT1080 cell conditioned medium, and shown to be the glycolytic enzyme, phosphoglycerate kinase (PGK). Recombinant PGK had the same specific activity as the fibrosarcoma derived protein.

Rate of secretion of PGK by carcinoma cells varies greatly (1-26 fold) among different carcinoma cell lines. Further, as outlined in Example 7 below, plasma of mice bearing fibrosarcoma tumours contained 6.6-fold more PGK than mice without tumours. In the present invention, administration of PGK to tumour-bearing individuals resulted in an increase in plasma levels of angiostatin containing free thiols, with subsequent inhibition of angiogenesis and tumour growth. These findings indicate that PGK not only functions in glycolysis but is secreted by tumour cells and participates in the angiogenic process as a disulfide reductase.

Methods of making PGK, or fragments thereof, can employ conventional techniques of molecular biology, microbiology, recombinant DNA and immunology, all of which are within the skill of the art and fully explained in for example, scientific publications, such as: "Molecular Cloning: A Laboratory Manual" Second Edition by Sambrook *et al.*, Cold Spring Harbor Press, 1989. For example, the gene for PGK (as

defined in SEQ ID NO:1) may be isolated from cells or tissues that express high levels of PGK by: isolating messenger RNA from the tissue or cells, subsequently using reverse transcriptase to generate the corresponding DNA sequence and finally using the polymerase chain reaction (PCR) with the appropriate primers to amplify the DNA sequence coding for the active PGK amino acid sequence. Also, a polynucleotide encoding a PGK fragment may be cloned into a expression vector, and then expressed in a suitable procaryotic, viral or eucaryotic host. Once expressed, PGK polypeptides can be purified according to standard procedures of the art, including: HPLC purification, size exclusion, ion-exchange and immuno-affinity (column) chromatography, gel electrophoresis and the like.

For instance, in producing recombinant PGK proteins of this invention, the DNA sequences of the invention are inserted into a suitable expression system. Preferably, a recombinant molecule or vector is constructed in which the polynucleotide sequence encoding PGK is operably linked to a heterologous expression control sequence permitting expression of the PGK protein. Numerous types of appropriate expression vectors are known in the art for mammalian (including human) protein expression, by standard molecular biology techniques. Such vectors may be selected from among conventional vector types including insects, such as baculovirus expression, or yeast, fungal, bacterial or viral expression systems.

Suitable host cells or cell lines for transfection in such a method include mammalian cells, such as Human 293 cells, Chinese hamster ovary cells (CHO), the monkey COS-1 cell line or murine 3T3 cells. Similarly bacterial cells are useful as host cells for the present invention. For example, the various strains of *E. coli* (e.g., HB101, MC1061) are well-known as host cells in the field, and various strains of *B. subtilis, Pseudomonas*, other bacilli and the like may also be employed. Also, many strains of yeast cells known to those skilled in the art are also available as host cells for expression of the polypeptides of the present invention. Alternatively, insect cells such as *Spodoptera frugipedera* (Sf9) cells may be used.

The vectors containing the DNA segments of interest can be transferred into the host cell by well-known methods, depending on the type of cellular host. For example, calcium chloride transfection and electroporation are commonly utilised for procaryotic cells, whereas calcium phosphate treatment, electroporation, lipofection, biolistics or viral-based transfection may be used for other cellular hosts. Other methods used to transform mammalian cells include the use of transfection, transformation, conjugation,

polybrene, liposomes, electroporation, particle gun technology and microinjection (see, generally, Sambrook *et al.*, 1989).

After introduction of the vector, recipient host cells are generally grown in a selective medium, which inherently selects for the growth of those cells containing the introduced vector. A variety of incubation conditions can be used to form the polypeptides of the present invention, but the most preferred conditions are those which mimic physiological.

Therefore, the present invention describes a method for producing a recombinant PGK protein which involves transfecting a host cell with at least one expression vector containing a recombinant polynucleotide encoding a PGK protein under the control of a transcriptional regulatory sequence. The transfected host cell is then cultured under conditions that allow expression of the PGK protein. The expressed protein is then recovered, isolated, and optionally purified from the culture medium (or from the cell, if expressed intracellularly) by appropriate means known to one of skill in the art. For example, the proteins may be isolated in soluble form following cell lysis, or may be extracted using known techniques, such as in guanidine chloride. Typically, the PGK protein of the invention may be produced as a fusion protein. For instance, it may be desirable to produce PGK fusion proteins, to enhance expression of the protein in a selected host cell, to improve purification, or for use in monitoring the presence of PGK in tissues, cells or cell extracts. Suitable fusion partners for the PGK protein of the invention are well known to those of skill in the art and include, among others, β-galactosidase, glutathione-S-transferase, and poly-histidine.

For example, microbial cells containing the exogenous PGK gene may be cultured in large volume reactors, collected by centrifugation and subsequently ruptured, for instance by high pressure homogenisation. The resulting cell lysate is resuspended in an appropriate diluent such as those described herein, and filtered to obtain an aqueous suspension of the PGK protein. For instance, the recombinant protein can be administered in crude form, for example, by diluting in a 0.1M phosphate buffer (pH 7.4) to 50-500 μg/ml concentration, and then passing through a sterile 0.22 micron filter.

PGK or fragments thereof may also be synthesised by standard methods of solid phase chemistry well known to those of ordinary skill in the art. For example, PGK fragments may be synthesised following the solid phase chemistry procedures of Steward and Young (Steward, J. M. & Young, J. D., Solid Phase Peptide Synthesis. (2nd Edn.) Pierce Chemical Co., Illinois, USA (1984).

In general, such a synthesis method comprises the sequential addition of one or more amino acids or suitably protected amino acids to a growing peptide chain. Typically, either the amino or carboxyl group of the first amino acid is protected by a suitable protecting group. The protected amino acid is then either attached to an inert solid support or utilised in solution by adding the next amino acid in the sequence having the complimentary (amino or carboxyl) group suitably protected and under conditions suitable for forming the amide linkage. The protecting group is then removed from this newly added amino acid residue and the next (protected) amino acid is added, and so forth. After all the desired amino acids have been linked, any remaining protecting groups, and if necessary any solid support, is removed sequentially or concurrently to produce the final polypeptide.

Amino acid changes in the PGK polypeptide or fragment thereof may be effected by techniques well known to those persons skilled in the relevant art. For example, amino acid changes may be effected by nucleotide replacement techniques which include the addition, deletion or substitution of nucleotides (conservative and/or non-conservative), under the proviso that the proper reading frame is maintained. Exemplary techniques include random mutagenesis, site-directed mutagenesis, oligonucleotide-mediated or polynucleotide-mediated mutagenesis, deletion of selected region(s) through the use of existing or engineered restriction enzyme sites, and the polymerase chain reaction.

An antibody (or fragment thereof) may be raised against PGK or an immunogenic portion thereof using the methods described below. In terms of obtaining a suitable amount, one may manufacture the antibody(s) using batch fermentation with serum free medium. After fermentation the antibody may be purified via a multistep procedure incorporating chromatography and viral inactivation/removal steps. For instance, the antibody may be first separated by Protein A affinity chromatography and then treated with solvent/detergent to inactivate any lipid enveloped viruses. Further purification, typically by anion and cation exchange chromatography may be used to remove residual proteins, solvents/detergents and nucleic acids. The purified antibody may be further purified and formulated into 0.9% saline using gel filtration columns. The formulated bulk preparation may then be sterilised and viral filtered and dispensed.

The administration of PGK as described in the present invention should be useful for treating angiogenesis-dependent diseases in vertebrates, especially cancer. Typically, the compounds of the invention may be useful for the treatment of angiogenesis-dependent diseases, such as cancer, solid tumours, hemangioma, arteriovenous

malformations, arthritis, Osler-Weber Syndrome, atherosclerotic plaques, psoriasis, corneal graft neovascularization, pyrogenic granuloma, wound healing, retrolental fibroplasia, diabetic retinopathy, scleroderma, granulations, angiofibroma, neovascular glaucoma, trachoma, hemophilic joints, hypertrophic scars, or gastric ulcers.

Typically, the cancer is a haematological tumour. More typically, the cancer is a solid tumour. Even more typically, the cancer includes both primary and metastatic solid tumours, and carcinomas of: oropharynx; hypopharynx; oesophagus; stomach; pancreas; liver; gallbladder; bile ducts; small intestine; urinary tract including kidney, bladder and urothelium; female genital tract including cervix, uterus, ovaries, choriocarcinoma and gestational trophoblastic disease; male genital tract including prostate, seminal vesicles, testes and germ cell tumours; endocrine glands including thyroid, adrenal, and pituitary; skin including hemangiomas, melanomas, sarcomas arising from bone or soft tissues and Kaposi's sarcoma; tumours of the brain, nerves, eyes, and meninges including astrocytomas, gliomas, glioblastomas, retinoblastomas, neuromas, neuroblastomas, Schwannomas and meningiomas; solid tumours arising from hematopoietic malignancies such as leukaemias, also chloromas, plasmacytomas, plaques and tumours of mycosis fungoides and cutaneous T-cell lymphoma/leukaemia; lymphomas including both Hodgkin's and non-Hodgkin's lymphomas.

For example, PGK should be useful for reducing tumour size, inhibiting tumour growth and/or prolonging the survival time of tumour-bearing vertebrates.

Typically, the vertebrate is selected from the group consisting of human, non-human primate, murine, bovine, ovine, equine, caprine, leporine, avian, feline and canine. More typically, the vertebrate is selected from the group consisting of human, non-human primate or murine. Even more typically, the vertebrate is human.

The pharmaceutical composition of the present invention is typically administered for therapeutic treatments. In a therapeutic application, compositions are administered to a patient already suffering from a disease, in an amount sufficient to cure or at least partially arrest the disease and its complications. Single or multiple administrations of the pharmaceutical compositions can be carried out with dose levels and pattern being selected by the treating physician. Regardless, the pharmaceutical composition of the present invention should provide a quantity of PGK sufficient to effectively treat the patient.

The therapeutically effective dose level for any particular patient will depend upon a variety of factors including: the disorder being treated and the severity of the

disorder; activity of the PGK or a fragment(s) or analogue thereof employed; the composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration; the route of administration; the rate of excretion of PGK; the duration of the treatment; drugs used in combination or coincidental with the PGK or a fragment(s) or analogue thereof, together with other related factors well known in medicine. For example, it is well known in the art to begin doses of a therapeutic compound, such as PGK, or PGK and at least one plasminogen activator, at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved.

Therefore, one skilled in the art would be able, by routine experimentation, to determine an effective, non-toxic amount of PGK which would be required to treat the disorders and diseases to which PGK is applicable. Generally, however, an effective dosage is expected to be in the range of about 0.0001 to about 100 mg PGK per kg body weight per 24 hours, preferably about 0.001 to about 100 mg PGK per kg body weight per 24 hours, more preferably about 0.01 mg to about 50 mg PGK per kg body weight per 24 hours, even more preferably about 0.1 mg to about 20 mg PGK per kg body weight per 24 hours, even more preferably still about 0.1 to about 10 mg PGK per kg body weight per 24 hours, and still even more preferably about 5 mg PGK per kg body weight per 24 hours. Further, if desired, the effective daily dose may be divided into multiple doses for purposes of administration.

Alternatively, an effective dosage may be up to about 500mg/m^2. Generally, an effective dosage is expected to be in the range of about 50 to about 500mg/m^2, preferably about 50 to about 350mg/m^2, more preferably about 50 to about 300mg/m^2, still more preferably about 50 to about 250mg/m^2, even more preferably about 75 to about 250mg/m^2, and still even more preferably about 75 to about 150mg/m^2.

Further, with respect to the administration of a plasminogen activator, an effective dosage is expected to be in the range of about 0.0001 to about 100 mg plasminogen activator per kg body weight per 24 hours, preferably about 0.001 to about 100 mg plasminogen activator per kg body weight per 24 hours, more preferably about 0.01 mg to about 50 mg plasminogen activator per kg body weight per 24 hours, even more preferably about 0.1 mg to about 20 mg plasminogen activator per kg body weight per 24 hours, even more preferably still about 0.1 to about 10 mg plasminogen activator

per kg body weight per 24 hours. Further, if desired, the effective daily dose may be divided into multiple doses for purposes of administration.

Alternatively, an effective dosage of the plasminogen activator may be up to about 500mg/m^2. Generally, an effective dosage is expected to be in the range of about 50 to about 500mg/m^2, preferably about 50 to about 350mg/m^2, more preferably about 50 to about 300mg/m^2, still more preferably about 50 to about 250mg/m^2, even more preferably about 75 to about 250mg/m^2, and still even more preferably about 75 to about 150mg/m^2.

Typically, the plasminogen activator is administered simultaneously with PGK in the same composition. Typically, the plasminogen activator is administered sequentially with PGK.

Typically the treatment would be for the duration of the condition, and contact times would typically be for the duration of the condition.

Further, it will be apparent to one of ordinary skill in the art that the optimal quantity and spacing of individual dosages of a compound of the present invention will be determined by the nature and extent of-the condition being treated, the form, route and site of administration, and the nature of the particular vertebrate being treated. Also, such optimum conditions can be determined by conventional techniques.

It will also be apparent to one of ordinary skill in the art that the optimal course of treatment, such as, the number of doses of the compound of the present invention given per day for a defined number of days, can be ascertained by those skilled in the art using conventional course of treatment determination tests.

Also included within the scope of the present invention are prodrugs of PGK and/or plasminogen activator. Typically, prodrugs will be functional derivatives of PGK, or PGK and a plasminogen activator, which are readily converted *in vivo* to the required compound for use in the present invention as described herein. Typical procedures for the selection and preparation of prodrugs are known to those of skill in the art and are described, for instance, in H. Bundgaard (Ed), *Design of Prodrugs*, Elsevier, 1985.

When used in the treatment of disease, the PGK, or PGK and at least one plasminogen activator compounds may be administered alone. However, it is generally preferable that PGK, or PGK and at least one plasminogen activator be administered in conjunction with other chemotherapeutic treatments conventionally administered to patients for treating disease. For example, a tumour may be treated conventionally with

surgery, radiation or chemotherapy and PGK, or PGK and at least one plasminogen activator, to extend the dormancy of micrometastases and to stabilise and inhibit the growth of any residual primary tumour.

Typically, when used in the treatment of solid tumours, compounds of the present invention may be administered with chemotherapeutic agents such as: adriamycin, taxol, fluorouricil, melphalan, cisplatin, alpha interferon, COMP (cyclophosphamide, vincristine, methotrexate and prednisone), etoposide, mBACOD (methortrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine and dexamethasone), PROMACE/MOPP (prednisone, methotrexate (w/leucovin rescue), doxorubicin, cyclophosphamide, taxol, etoposide/mechlorethamine, vincristine, prednisone and procarbazine), vincristine, vinblastine, angioinhibins, TNP-470, pentosan polysulfate, platelet factor 4, angiostatin, LM-609, SU-101, CM-101, Techgalan, thalidomide, SP-PG and the like. Other chemotherapeutic agents include alkylating agents such as nitrogen mustards including mechloethamine, melphan, chlorambucil, cyclophosphamide and ifosfamide; nitrosoureas including carmustine, lomustine, semustine and streptozocin; alkyl sulfonates including busulfan; triazines including dacarbazine; ethyenimines including thiotepa and hexamethylmelamine; folic acid analogues including methotrexate; pyrimidine analogues including 5-fluorouracil, cytosine arabinoside; purine analogues including 6-mercaptopurine and 6-thioguanine; antitumour antibiotics including actinomycin D; the anthracyclines including doxorubicin, bleomycin, mitomycin C and methramycin; hormones and hormone antagonists including tamoxifen and cortiosteroids and miscellaneous agents including cisplatin and brequinar.

In general pharmaceutical formulations of the present invention may be prepared according to methods which are known to those of ordinary skill in the art and accordingly may include a pharmaceutically acceptable carrier, diluent and/or adjuvant.

These formulations (PGK or PGK and at least one plasminogen activator) can be administered by standard routes. In general, the combinations may be administered by the topical, transdermal, intraperitoneal, intracranial, intracerebroventricular, intracerebral, intravaginal, intrauterine, oral, rectal or parenteral (e.g., intravenous, intraspinal, subcutaneous or intramuscular) route. In addition, PGK, or PGK and at least one plasminogen activator may be incorporated into biodegradable polymers allowing for sustained release, the polymers being implanted in the vicinity of where drug delivery is desired, for example, at the site of for example, a tumour or implanted so that the active agents are slowly released systemically. Osmotic minipumps may also be used to provide

controlled delivery of high concentrations of the active agents through cannulae to the site of interest, such as directly into for example, a metastatic growth or into the vascular supply to that tumour.

The carriers, diluents and adjuvants must be "acceptable" in terms of being compatible with the other ingredients of the formulation, and not deleterious to the recipient thereof.

Examples of pharmaceutically and veterinarily acceptable carriers or diluents are demineralised or distilled water; saline solution; vegetable based oils such as peanut oil, safflower oil, olive oil, cottonseed oil, maize oil, sesame oils such as peanut oil, safflower oil, olive oil, cottonseed oil, maize oil, sesame oil, arachis oil or coconut oil; silicone oils, including polysiloxanes, such as methyl polysiloxane, phenyl polysiloxane and methylphenyl polysolpoxane; volatile silicones; mineral oils such as liquid paraffin, soft paraffin or squalane; cellulose derivatives such as methyl cellulose, ethyl cellulose, carboxymethylcellulose, sodium carboxymethylcellulose or hydroxypropylmethylcellulose; lower alkanols, for example ethanol or iso-propanol; lower aralkanols; lower polyalkylene glycols or lower alkylene glycols, for example polyethylene glycol, polypropylene glycol, ethylene glycol, propylene glycol, 1,3-butylene glycol or glycerin; fatty acid esters such as isopropyl palmitate, isopropyl myristate or ethyl oleate; polyvinylpyrridone; agar; carrageenan; gum tragacanth or gum acacia, and petroleum jelly. Typically, the carrier or carriers will form from 10% to 99.9% by weight of the compositions.

In a preferred form the pharmaceutical composition of the invention comprises an effective amount of PGK, or PGK and at least one plasminogen activator together with a pharmaceutically acceptable carrier, diluent and/or adjuvant as shown in Examples 10 or 11.

The pharmaceutical composition of the invention may be in the form of a composition in a form suitable for administration by injection, in the form of a capsule suitable for oral ingestion, in the form of an ointment, cream or lotion suitable for topical administration, in a form suitable for delivery as an eye drop, in an aerosol form suitable for administration by inhalation, such as by intranasal inhalation or oral inhalation, in a form suitable for parenteral administration, that is, subcutaneous, intramuscular or intravenous injection.

For administration as an injectable solution or suspension, non-toxic parenterally acceptable diluents or carriers can include, Ringer's solution, isotonic saline, phosphate buffered saline, ethanol and 1,2 propylene glycol.

Some examples of suitable carriers, diluents, excipients and adjuvants for oral use include peanut oil, liquid paraffin, sodium carboxymethylcellulose, methylcellulose, sodium alginate, gum acacia, gum tragacanth, dextrose, sucrose, sorbitol, mannitol, gelatine and lecithin. In addition these oral formulations may contain suitable flavouring and colourings agents. When used in capsule form the capsules may be coated with compounds such as glyceryl monostearate or glyceryl distearate which delay disintegration.

Adjuvants typically include emollients, emulsifiers, thickening agents, preservatives, bactericides and buffering agents.

Solid forms for oral administration may contain binders acceptable in human and veterinary pharmaceutical practice, sweeteners, disintegrating agents, diluents, flavourings, coating agents, preservatives, lubricants and/or time delay agents. Suitable binders include gum acacia, gelatine, corn starch, gum tragacanth, sodium alginate, carboxymethylcellulose or polyethylene glycol. Suitable sweeteners include sucrose, lactose, glucose, aspartame or saccharine. Suitable disintegrating agents include corn starch, methylcellulose, polyvinylpyrrolidone, guar gum, xanthan gum, bentonite, alginic acid or agar. Suitable diluents include lactose, sorbitol, mannitol, dextrose, kaolin, cellulose, calcium carbonate, calcium silicate or dicalcium phosphate. Suitable flavouring agents include peppermint oil, oil of wintergreen, cherry, orange or raspberry flavouring. Suitable coating agents include polymers or copolymers of acrylic acid and/or methacrylic acid and/or their esters, waxes, fatty alcohols, zein, shellac or gluten. Suitable preservatives include sodium benzoate, vitamin E, alpha-tocopherol, ascorbic acid, methyl paraben, propyl paraben or sodium bisulphite. Suitable lubricants include magnesium stearate, stearic acid, sodium oleate, sodium chloride or talc. Suitable time delay agents include glyceryl monostearate or glyceryl distearate.

Liquid forms for oral administration may contain, in addition to the above agents, a liquid carrier. Suitable liquid carriers include water, oils such as olive oil, peanut oil, sesame oil, sunflower oil, safflower oil, arachis oil, coconut oil, liquid paraffin, ethylene glycol, propylene glycol, polyethylene glycol, ethanol, propanol, isopropanol, glycerol, fatty alcohols, triglycerides or mixtures thereof.

Suspensions for oral administration may further comprise dispersing agents and/or suspending agents. Suitable suspending agents include sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethyl-cellulose, poly-vinyl-pyrrolidone, sodium alginate or acetyl alcohol. Suitable dispersing agents include lecithin, polyoxyethylene esters of fatty acids such as stearic acid, polyoxyethylene sorbitol mono- or di-oleate, -stearate or -laurate, polyoxyethylene sorbitan mono- or di-oleate, -stearate or -laurate and the like.

The emulsions for oral administration may further comprise one or more emulsifying agents. Suitable emulsifying agents include dispersing agents as exemplified above or natural gums such as guar gum, gum acacia or gum tragacanth.

Methods for preparing parenterally administrable compositions are apparent to those skilled in the art, and are described in more detail in, for example, Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pa., hereby incorporated by reference herein.

The topical formulations of the present invention, comprise an active ingredient together with one or more acceptable carriers, and optionally any other therapeutic ingredients. Formulations suitable for topical administration include liquid or semi-liquid preparations suitable for penetration through the skin to the site of where treatment is required, such as liniments, lotions, creams, ointments or pastes, and drops suitable for administration to the eye, ear or nose.

Drops according to the present invention may comprise sterile aqueous or oily solutions or suspensions. These may be prepared by dissolving the active ingredient in an aqueous solution of a bactericidal and/or fungicidal agent and/or any other suitable preservative, and optionally including a surface active agent. The resulting solution may then be clarified by filtration, transferred to a suitable container and sterilised. Sterilisation may be achieved by: autoclaving or maintaining at 90°C-100°C for half an hour, or by filtration, followed by transfer to a container by an aseptic technique. Examples of bactericidal and fungicidal agents suitable for inclusion in the drops are phenylmercuric nitrate or acetate (0.002%), benzalkonium chloride (0.01%) and chlorhexidine acetate (0.01%). Suitable solvents for the preparation of an oily solution include glycerol, diluted alcohol and propylene glycol.

Lotions according to the present invention include those suitable for application to the skin or eye. An eye lotion may comprise a sterile aqueous solution optionally containing a bactericide and may be prepared by methods similar to those described

above in relation to the preparation of drops. Lotions or liniments for application to the skin may also include an agent to hasten drying and to cool the skin, such as an alcohol or acetone, and/or a moisturiser such as glycerol, or oil such as castor oil or arachis oil.

Creams, ointments or pastes according to the present invention are semi-solid formulations of the active ingredient for external application. They may be made by mixing the active ingredient in finely-divided or powdered form, alone or in solution or suspension in an aqueous or non-aqueous fluid, with a greasy or non-greasy basis. The basis may comprise hydrocarbons such as hard, soft or liquid paraffin, glycerol, beeswax, a metallic soap; a mucilage; an oil of natural origin such as almond, corn, arachis, castor or olive oil; wool fat or its derivatives, or a fatty acid such as stearic or oleic acid together with an alcohol such as propylene glycol or macrogols.

The formulation may incorporate any suitable surfactant such as an anionic, cationic or non-ionic surfactant such as sorbitan esters or polyoxyethylene derivatives thereof. Suspending agents such as natural gums, cellulose derivatives or inorganic materials such as silicaceous silicas, and other ingredients such as lanolin, may also be included.

The pharmaceutical compositions of the invention may also be administered in the form of liposomes. Liposomes are generally derived from phospholipids or other lipid substances, and are formed by mono- or multi-lamellar hydrated liquid crystals that are dispersed in an aqueous medium. Any non-toxic, physiologically acceptable and metabolisable lipid capable of forming liposomes can be used. The formulations of the present invention in liposome form may contain, in addition to a compound of the present invention, stabilisers, preservatives, excipients and the like. The preferred lipids are the phospholipids and the phosphatidyl cholines (lecithins), both natural and synthetic. Methods to form liposomes are known in the art, and in relation to this specific reference is made to: Prescott, Ed., Methods in Cell Biology, Volume XIV, Academic Press, New York, N.Y. (1976), p. 33 *et seq.*, the contents of which is incorporated herein by reference.

PGK can also be used to generate antibodies that are specific for PGK, wherein these antibodies can include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library. A molecule is "antigenic" when it is capable of specifically interacting with an antigen recognition molecule of the immune system, such as an immunoglobulin (antibody) or T cell antigen receptor. Therefore, an antigenic PGK polypeptide contains at least about 5, and preferably at least

about 10, amino acids. Further, the antigenic portion of a PGK molecule can be that portion that is immunodominant for antibody or T cell receptor recognition, or it can be that portion used to generate an antibody to the molecule by conjugating the antigenic portion to a carrier molecule for immunization. Importantly, a PGK molecule that is antigenic need not be itself immunogenic, that is, capable of eliciting an immune response without a carrier.

A monoclonal antibody refers to an antibody having only one species of antibody combining site capable of immunoreacting with a particular antigen. Therefore, a monoclonal antibody displays a single binding affinity for any antigen with which it immunoreacts.

PGK antibodies are raised using methods well known to those skilled in the art. For instance, a PGK monoclonal antibody, typically containing Fab portions, may be prepared using the hybridoma technology described in Antibodies-A Laboratory Manual, Harlow and Lane, eds., Cold Spring Harbor Laboratory, N.Y. (1988), the disclosure of which is incorporated herein by reference.

In essence, in the preparation of monoclonal antibodies directed toward PGK polypeptide, or fragment, analogue, or derivative thereof, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include the hybridoma technique originally developed by Kohler et al., Nature, 256:495-497 (1975), as well as the trioma technique, the human B-cell hybridoma technique [Kozbor *et al.*, Immunology Today, 4:72 (1983)], and the EBV-hybridoma technique to produce human monoclonal antibodies [Cole *et al.*, in Monoclonal Antibodies and Cancer Therapy, pp. 77-96, Alan R. Liss, Inc., (1985)]. Immortal, antibody-producing cell lines can be created by techniques other than fusion, such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, e.g., M. Schreier *et al.*, "Hybridoma Techniques" (1980); Hammerling et al., "Monoclonal Antibodies and T-cell Hybridomas" (1981); Kennett *et al.*, "Monoclonal Antibodies" (1980); wherein the disclosures of each of these citations are also incorporated herein by reference.

In summary, a means of producing a hybridoma from which the monoclonal antibody is produced, a myeloma or other self-perpetuating cell line is fused with lymphocytes obtained from the spleen of a mammal hyperimmunised with a recognition factor-binding portion thereof, or recognition factor, or an origin-specific DNA-binding portion thereof. Hybridomas producing a monoclonal antibody useful in practicing this

invention are identified by their ability to immunoreact with the present recognition factor and their ability to inhibit specified transcriptional activity in target cells.

A monoclonal antibody useful in practicing the present invention can be produced by initiating a monoclonal hybridoma culture comprising a nutrient medium containing a hybridoma that secretes antibody molecules of the appropriate antigen specificity. The culture is maintained under conditions and for a time period sufficient for the hybridoma to secrete the antibody molecules into the medium. The antibody-containing medium is then collected. The antibody molecules can then be further isolated by well-known techniques.

Media useful for the preparation of these compositions are both well-known in the art and commercially available and include synthetic culture media, inbred mice etc. An exemplary synthetic medium is Dulbecco's minimal essential medium (DMEM; Dulbecco et al., Virol. 8:396 (1959)) supplemented with 4.5 gm/l glucose, 20 mm glutamine, and 20% foetal calf serum. An exemplary inbred mouse strain is the Balb/c.

Similarly, there are various procedures known in the art which may be used for the production of polyclonal antibodies to PGK, or fragments thereof. For the production of PGK polyclonal antibody, various host animals can be immunized by injection with the PGK polypeptide, or a derivative (e.g., fragment or fusion protein) thereof, including but not limited to rabbits, mice, rats, sheep, goats, etc. Further, the PGK polypeptide or fragment thereof can be conjugated to an immunogenic carrier, e.g., bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). Also, various adjuvants may be used to increase the immunological response, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminium hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*.

Screening for the desired PGK antibody can also be accomplished by techniques known in the art, and these include radioimnmunoassay, ELISA (enzyme-linked immunosorbent assay), sandwich imnmunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immnunodiffusion assays, in situ immunoassays, Western blots, precipitation reactions, agglutination assays, complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, and the like.

These antibodies can be used in diagnostic methods and kits that are well known to those of ordinary skill in the art to detect or quantify PGK in a body fluid or tissue, and results from these tests can be used to diagnose or predict the occurrence or recurrence of a cancer and other angiogenic mediated diseases.

The antibody (or fragment thereof) raised against PGK or a fragment(s) thereof has binding affinity for PGK. Preferably, the antibody (or fragment thereof) has binding affinity or avidity greater than about 10^5 M^{-1}, more preferably greater than about 10^6 M^{-1}, more preferably still greater than about 10^7 M^{-1} and most preferably greater than about 10^8 M^{-1}.

Further to this, the present invention encompasses a method of detecting disease, wherein said method comprises detecting a PGK polypeptide, said method comprises:

(a) contacting a sample with an antibody (or fragment thereof) raised against PGK, and

(b) detecting the presence of the antibody (or fragment thereof) bound to PGK polypeptide.

Typically, PGK is present in the plasma of vertebrates. More typically, plasma PGK levels correlate with disease progression, wherein an example of such a disease is cancer. Even more typically, plasma of healthy vertebrates contains little or no PGK.

Also, patients having circulating anti-PGK antibodies may well have a greater likelihood of developing multiple tumours and cancers, and may be more likely to have recurrences of cancer after treatments or periods of remission. The Fab fragments of these anti-PGK antibodies can be used as antigens to generate anti-PGK Fab-fragment antisera which can be used to neutralise anti-PGK antibodies. Such a method would reduce the removal of circulating PGK by anti-PGK antibodies, thereby effectively elevating circulating PGK levels, and thus increase the level of the angiogenesis inhibitor -angiostatin.

Another aspect of the present invention is a method of blocking the action of excess endogenous PGK. This can be done by passively immunising a vertebrate with antibodies specific for PGK. For example, this treatment can be important in treating abnormal vasculogenesis, thereby providing a useful tool to examine the effects of PGK removal on metastatic processes. The Fab fragment of PGK antibodies contains the binding site for PGK, and this fragment can be isolated from PGK antibodies using techniques known to those skilled in the art, wherein these fragments are then used as antigens to generate production of anti-Fab fragment serum.

Infusion of this antiserum against the Fab fragments of PGK prevents PGK from binding to PGK antibodies. Consequently, therapeutic benefit is obtained by neutralising endogenous anti-PGK antibodies, wherein the net effect is to facilitate the ability of endogenous circulating PGK to reach target cells, thereby decreasing the spread of metastases.

Conditions for incubating an antibody (or fragment thereof) with a test sample vary widely, depending on the format of detection used in the assay, the detection method, and the type and nature of the antibody used. A person of ordinary skill in the art would readily appreciate that any one of the commonly available immunological assays could be used in performing the method of detection. For example, these assays include: radioimmunoassays, enzyme-linked immunosorbent assays, and/or immunoflourescent assays.

Further, the test sample used in the assay may consist of tissue, cells, protein or membrane extracts of cells, and biological fluids, such as blood, serum, plasma or urine.

A kit for performing the above method of the invention contains all the necessary reagents to carry out the above methods of detection. For example, the kit may comprise the following containers:

(a) a first container containing the antibody (or fragment thereof) raised against PGK;

(b) a second container containing a conjugate comprising a binding partner of the antibody (or fragment thereof), together with a detectable label.

Typically, the kit may further comprise one or more other containers, containing other components, such as wash reagents, and other reagents capable of detecting the presence of bound antibodies. More typically, the detection reagents may include: labelled (secondary) antibodies, or where the antibody (or fragment thereof) raised against PGK is itself labelled, the compartments may comprise antibody binding reagents capable of reacting with the labelled antibody (or fragment thereof) raised against PGK.

In detail, a compartmentalised kit includes any kit in which reagents are contained in separate containers. Such containers include small glass containers, plastic containers or strips of plastic or paper. Such containers allow the efficient transfer of reagents from one compartment to another compartment such that the samples and reagents are not cross-contaminated and the agents or solutions of each container can be added in a quantitative fashion from one compartment to another. Such containers will include a container which will accept the test sample, a container which contains the

probe or primers used in the assay, containers which contain wash reagents (such as phosphate buffered saline, Tris-buffers, and like), and containers which contain the reagent detect the bound antibody, amide product, or the like.

These assay kits include, but are not limited to the following techniques: competitive and non-competitive assays, radioimmunoassay, bioluminescence and chemiluminescence assays, fluorometric assays, sandwich assays, immunoradiometric assays, dot blots, enzyme linked assays including ELISA, microtiter plates, antibody coated strips or dipsticks for rapid monitoring of urine or blood, and immunocytochemistry.

An example of one such kit is used in the localisation of PGK in tissues and cells. This kit includes: PGK antibodies, and secondary antibodies linked to a fluorescent molecule such as fluorescein isothiocyanate, or to some other reagent used to visualise the primary PGK antibody. Such a PGK kit permits localisation of PGK in tissue sections and cultured cells using both light and electron microscopy. For example, tumors are biopsied or collected and tissue sections cut with a microtome to examine sites of PGK production. Such information is useful for diagnostic and possibly therapeutic purposes in the detection and treatment of cancer.

In yet another aspect of the present invention is a method of gene therapy for the treatment of disease, primarily in the inhibition of angiogenesis. In its simplest form, gene transfer can be performed by simply injecting minute amounts of DNA into the nucleus of a cell, through a process of microinjection. Once recombinant genes are introduced into a cell, they can be recognised by the cells normal mechanisms for transcription and translation, and a gene produce will be expressed.

A number of methods have also been attempted for introducing DNA into larger numbers of cells. These methods include: transfection, wherein DNA is precipitated with $CaPO_4$ and taken into cells by pinocytosis; electroporation, wherein cells are exposed to large voltage pulses to introduce holes into the membrane; lipofection/liposome fusion, wherein DNA is packaged into lipophilic vesicles which fuse with a target cell; and particle bombardment using DNA bound to small projectiles. Another method for introducing DNA into cells is to couple the DNA to chemically modified proteins.

For example, an expression vector containing a nucleic acid molecule encoding for a phosphoglycerate kinase, or a fragment(s) or analogue thereof, or a vector comprising a nucleic acid molecule encoding for said a phosphoglycerate kinase, or a fragment(s) or analogue thereof, is inserted into cells, the cells are grown *in vitro* and then infused in

large numbers into patients. Expression vectors derived from viruses such as retroviruses, vaccinia virus, adenovirus, adeno-associated virus, herpes viruses, several RNA viruses, or bovine papilloma virus, may be used for delivery of these nucleic acid sequences into the targeted cell population (eg., tumour cells). Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors containing coding sequences. Alternatively, recombinant nucleic acid molecules encoding protein sequences can be used as naked DNA or in reconstituted system, for example, liposomes or other lipid systems for delivery to target cells.

For instance, it has also been shown that adenovirus proteins are capable of destabilising endosomes and enhancing the uptake of DNA into cells. The admixture of adenovirus to solutions containing DNA complexes, or the binding of DNA to polylysine covalently attached to adenovirus using protein crosslinking agents substantially improves the uptake and expression of the recombinant gene.

The invention will now be described in greater detail by reference to specific Examples, which should not be construed as in any way limiting the scope of the invention.

Examples

Example 1

Assay and Purification of Plasmin Reductase

Plasmin reductase activity in the conditioned medium of transformed cells was measured from formation of free thiols in angiostatin using the avidin-biotin interaction in a microtitre plate format. Briefly, plasmin was incubated with samples containing plasmin reductase and the resulting angiostatin fragments labeled with the biotin-linked maleimide, 3-(N-maleimidylpropionyl)biocytin (MPB).

Firstly, plasminogen was purified from fresh frozen human plasma and separated into its two carbohydrate variants according to published procedures [F. J. Castellino and J. R. Powell, *Methods Enzymol.* 80, 365 (1981)]. Plasmin was made from plasminogen using urokinase plasminogen activator as described previously (3). Samples were incubated with 2 μg per ml of plasmin in of 20 mM Hepes, 0.14 M NaCl, 0.05% Tween 20, pH 7.4 buffer (Hepes/Tween) for 30 min at 37°C. The control reaction was plasmin incubated in Hepes/Tween. The angiostatin fragments formed were labeled with MPB (100 μM) (Molecular Probes, Palo Alto, CA USA) for 30 minutes at room temperature, followed by quenching of the unreacted MPB with reduced glutathione (GSH) (200 μM)

for 10 minutes at room temperature. Unreacted GSH, and other free sulfhydryls in the system, were blocked with iodoacetamide (400 μM) for 10 min at room temperature.

The MPB-labeled angiostatin fragments were adsorbed to microtitre plate wells coated with an anti-angiostatin monoclonal antibody and the incorporated MPB measured by incubation with peroxidase-conjugated streptavidin and a chromogenic substrate (Figure 1A).

More specifically, a murine monoclonal antibody was generated against human angiostatin (4) as previously described in P. A. Underwood and P. A. Bean, *J. Immunol. Methods* 107, 119 (1988). The antibody, designated 8.19, binds with equivalent affinity to soluble angiostatin and plasmin and with approximately 10-fold lower affinity to plasminogen. The 8.19 antibody (100 μl of 5 μg per ml in 0.1 M $NaHCO_3$, 0.02 % NaN_3, pH 8.3) was adsorbed to Nunc PolySorp 96 well plates overnight at 4°C in a humid environment. Wells were washed once with Hepes/Tween, non-specific binding sites blocked by adding 200 μl of 2% BSA in 20 mM Hepes, 0.14 M NaCl, 0.02 % NaN_3, pH 7.4 buffer and incubating for 90 minutes at 37°C, and then washed two times with Hepes/Tween. MPB-labeled angiostatin fragments were diluted 1:10 to 1:1000 in Hepes/Tween and 100 μl aliquots added to antibody coated wells and incubated for 30 minutes at room temperature with orbital shaking. Wells were washed three times with Hepes/Tween and 100 μl of 1:100 dilution of StreptABComplex/HRP (Dako Carpenteria, CA USA) in Hepes/Tween added and incubated for 30 minutes at room temperature with orbital shaking. Wells were washed three times with Hepes/Tween and the colour developed with 100 μl of 0.003% H_2O_2, 1 mg per ml 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma, St Louis, MO USA) in 50 mM citrate, pH 4.5 buffer for 20 minutes at room temperature with orbital shaking. Absorbances were read at 405 nm using a Molecular Devices Thermomax Kinetic Microplate Reader (Molecular Devices Corporation, CA USA). Results were corrected for control wells not incubated with MPB-labeled angiostatin.

Plasmin was incubated with samples containing plasmin reductase and the resulting angiostatin fragments labeled with the thiol-specific biotin-linked maleimide, MPB. The MPB-labeled angiostatin fragments were bound to an anti-angiostatin monoclonal antibody coated on microtitre plate wells and the incorporated MPB measured by incubation with peroxidase-conjugated streptavidin and a chromogenic substrate (Fig. 1a). There was a linear relationship ($r^2 = 0.99$) between plasmin reductase concentration and

absorbance value after a threshold concentration of plasmin reductase was achieved (Figure 1B). Plasmin reductase activity was measured in this linear range.

Example 2

Purification of Plasmin Reductase

It was observed that plasmin reductase activity in HT1080 fibrosarcoma conditioned medium was partially inhibited by the adenine nucleotides, NAD(H) or ATP, and inactivated by the thiol modifying reagents, iodoacetamide or N-ethylmaleimide (not shown). These results implied that plasmin reductase would have affinity for the chomatography matrices, Cibachron Blue-Sepharose and activated thiopropyl-Sepharose.

Specifically, a conditioned medium of HT1080 cells was prepared using Nunc Cell Factories. HT1080 cells (approximately 100,000 cells per cm^2 of cell factory area) were seeded into cell factories in DMEM with 10% foetal calf serum (GibCo BRL, Granc Island, NY). When the cells were at ~80% confluence they were washed twice with phosphate-buffered saline and incubated with Hank's balanced salt solution at 37°C, 5% CO_2 for 30 hours. The conditioned medium was collected, centrifuged at 1200 g for 10 minutes and passed through a 0.2 μM filter to remove detached cells and cellular debris, and stored at -20°C. Twenty litres of conditioned medium was concentrated to 350 mls using a Amicon spiral-wound concentrator with a 10 kDa cutoff membrane. The proteinase inhibitors, leupeptin (10 μM), PMSF (1 mM), EDTA (2 mM) and soybean trypsin inhibitor (10 μg per ml) were added to the concentrated medium to minimise proteolytic degradation of the plasmin reductase.

The concentrated medium was dialyzed (2 x 4 litres) against 20 mM Hepes, 0.05 M NaCl, 1 mM EDTA, 0.02% NaN_3, pH 7.4 buffer and applied to a 80 ml column of Cibachron Blue-Sepharose (2.5 x 17 cm) (Pharmacia Biotech, Upsalla Sweden) equilibrated with the same buffer. The column was washed with 3 bed volumes of the Hepes buffer at a flow rate of 0.5 ml per min to elute unbound proteins and developed with a 240 ml linear NaCl gradient from 0.05 to 2 M in the Hepes buffer. Plasmin reductase activity eluted at ~1 M NaCl (Figure 1C).

The fractions containing plasmin reductase activity (~17 mls) were dialyzed (3 x 1 litre) against 20 mM Hepes, 0.14 M NaCl, 1 mM EDTA, pH 7.4 buffer and batch adsorbed for 60 minutes at 20°C to 6 ml of swollen thiopropyl-Sepharose (Pharmacia Biotech, Upsalla Sweden) equilibrated with the same buffer. The matrix was packed into a column (1 x 8 cm) and washed with 10 bed volumes of the equilibration buffer at a flow

rate of 0.1 ml per minute. Proteins were eluted with buffers of increasing reducing potential. The buffers were 20 mM Hepes, 0.14 M NaCl, 1 mM EDTA, pH 7.4 containing either 5 mM cysteine, 50 mM GSH or 20 mM dithiothreitol. One void volume (2 mls) of buffer containing reducing agent was run into the column and allowed to react with the matrix for 30 minutes at 20°C before eluting the protein with 10 mls of buffer without reducing agent. Plasmin reductase activity was eluted with dithiothreitol (2mg with 710-fold increase in specific activity).

The eluate was concentrated to 2.4 ml, dialyzed (2 x 2 litres) against 20 mM Hepes, 0.14 M NaCl, 1 mM EDTA, 0.02% NaN_3, pH 7.4 buffer and applied to a 120 ml column of Sephacryl S-200 HR (1.5 x 70 cm) at a flow rate of 0.2 ml per minute. The plasmin reductase activity eluted at ~70 mls (Figure 1D).

The plasmin reductase activity was well resolved and associated with a single polypeptide chain with a molecular mass of ~42 kDa by SDS-PAGE (Figure 2C). Approximately 20 μg of plasmin reductase was isolated with a 5,000-fold increase in specific activity.

Example 3

Identification of plasmin reductase as phosphoglycerate kinase

The intact 42 kDa protein was refractive to N-terminal sequencing. The protein was digested with CNBr, the fragments resolved by reverse-phase HPLC on a Vydac C8 column using a linear acetonitrile/0.1% trifluoracetic acid gradient, and 34 N-terminal residues of 4 internal peptides were determined using a Hewlett-Packard G1000A protein sequencing system. Plasmin reductase was found to have 100% sequence identity to phosphoglycerate kinase (8) (PGK; ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) (Fig. 2).

Example 4

Cloning of PGK and production of recombinant protein

Human PGK cDNA was cloned by RT-PCR from HT1080 RNA. A 1.33 kb hPGK cDNA was isolated by RT-PCR from total RNA extracted from HT1080 cells. The forward and reverse primers were 5'-AGTACATATGTCGCTTTCTAACAAGCTG-3' (positions 80 to 100) and 5'-AGTAGGATCCCTAATGCCAAGTGGAGATGCA-3' (positions 1409 to 1389), respectively. A NdeI restriction site in the forward primer and a BamHI site in the reverse primer were incorporated to facilitate cloning. The hPGK cDNA contained the open reading frame of hPGK which codes for 418 amino acids.

RNA and DNA purification kits were from Qiagen, DNA polymerase Pfx was from Life Technologies, and restriction enzymes were from Roche. Integrity of the cDNA was confirmed by automatic sequencing (ABI-377 Automatic Sequencer, Applied Biosystems) and was the same as the reported sequence (8).

The hPGK cDNA was sub-cloned into the plasmid vector, pET11a (pET11a-hPGK), which was then transfected into *E. coli* strain, BL21 (DE3) (Novagen). The transfected cells were selected and grown in LB medium containing 75 mg per ml ampicillin to an optical density at 600 nm of 0.8. Expression of recombinant protein was induced by 0.5 mM IPTG for 5 hours at 37°C. The Bl21 cells were collected by centrifugation at 5000 g for 15 minutes and resuspended in B-PER Bacterial Extraction Reagent at a ratio of 60 mls per litre of culture (Pierce, Rochford, IL). The cells were lysed by vortexing for 3 minutes and gentle shaking for a further 10 minutes. The lysate was clarified by centrifugation at 14,000 g for 30 minutes. Solid $(NH_4)_2SO_4$ was added slowly to the clarified supernatant to a concentration of 25% and stirred for 20 minutes at 4°C. The pellet was collected by centrifugation at 12,000 g for 20 minutes and additional $(NH_4)_2SO_4$ added to the supernatant to a final concentration of 100% and stirred for 20 minutes at 4°C. The pellet was collected by centrifugation at 12,000 g for 20 minutes, dissolved in 20 mM Hepes, 0.05 M NaCl, 1 mM EDTA, 0.02% NaN_3, pH 7.4 buffer and dialyzed against the same buffer (3 x 2 litres). The dialyzed protein was applied to a 80 ml column of Cibachron Blue-Sepharose (2.5 x 17 cm) equilibrated with the same Hepes buffer. The column was washed with 3 bed volumes of the Hepes buffer at a flow rate of 0.5 ml per min to elute unbound proteins and developed with a 240 ml linear NaCl gradient from 0.05 to 2 M in the Hepes buffer. Plasmin reductase activity eluted at ~ 1 M NaCl. The fractions containing plasmin reductase activity (~20 mls) were dialyzed (2 x 1 litre) against 20 mM MES, 1 mM EDTA, pH 6.0 buffer and applied to a 8 ml column of Q Sepharose Fast Flow (Pharmacia Biotech, Upsalla, Sweden) (1 x 10 cm) at a flow rate of 0.5 ml per min. The matrix was washed with the MES buffer to elute the unbound protein which contained all the plasmin reductase activity. The flow through was dialyzed against degassed water (3 x 1 litre) and lyophilised.

Recombinant human PGK (rhPGK) resolved at the same size as the HT1080 protein on SDS-PAGE (Fig. 3a) and had the same plasmin reductase activity as the HT1080 protein (Fig. 3b and c). These findings demonstrate that plasmin reductase is PGK. Rabbit muscle PGK had comparable specific plasmin reductase activity to rhPGK or HT1080 PGK while yeast PGK had negligible activity (Fig. 3c). Human and rat PGK

share 85% sequence identity (97% sequence homology) while the human and yeast enzyme share only 65% sequence identity.

Example 5

Cloning and activity of the C-terminal domain of PGK

A *Nde*I site was incorporated at codon 190 in pET11a-hPGK by site-directed mutagenesis (Quikchange II mutagenesis kit, Integrated Sciences). The forward and reverse primers for mutagenesis were 5'-GCTGGTGGGTTTTTGCATATGAAGAAGGAGCTG-3' and 5'-CAGCTCCTTCTTCATATGCAAAAACCCACCAGC-3'. Codons 1 to 189 were deleted by *Nde*I digestion and re-ligation and the ATG at position 190 was used as the start codon. A C-terminal peptide of 23 kDa (amino acids 190 to 417) was expressed and purified as for the full length PGK (see Example 4).

The recombinant C-terminal PGK fragment resolved at the appropriate size on SDS-PAGE (Fig. 4a) and had ~35% of the plasmin reductase activity of full length PGK (Fig. 4b).

Example 6

Secretion of PGK from carcinoma cells

PGK concentration was measured by formation of 1,3-bisphospho-D-glycerate from 3-phospho-D-glycerate in a coupled assay with glyceraldehyde-3-phosphate dehydrogenase (Kuntz & Krietsch, (1982) *Methods Enzymol.* 90, 103). The reaction was in 50 mM Hepes, 50 mM KCl, 0.2 mM EDTA, 0.075 M NaCl, pH 7.5 buffer containing 1.2 mM Mg^{2+}, 150 μM NADH, 10 mM ATP, 10 mM 3-phospho-D-glycerate and 2 μg per ml rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. The initial rate of oxidation of NADH was measured from decrease in absorbance at 340 nm using a Molecular Devices SPECTRAmax PLUS kinetic microplate reader. The rates were corrected for background NADH oxidation in reactions containing all components except 3-phospho-D-glycerate. The concentration of PGK in the samples was extrapolated from a standard curve constructed using known concentrations of rhPGK. Serum-free conditioned medium from carcinoma cells was collected for 24 hours and the weight of PGK secreted from one million cells determined.

Rates of secretion of PGK by carcinoma cells varied considerably (Fig. 5). Cultured HT1080 cells secreted 0.43 ± 0.01 μg of PGK per 10^6 cells in 24 hours. This rate of secretion was compared with rates of secretion of PGK from four human

pancreatic (AsPC-1, BxPC-3, SU.86.86 and Hs766T), three human breast (BT20, MDA-231 and MCF-7M) and three human colon (Lim1215, Lim2412 and Lim1863) carcinoma cell lines. The secretion rates varied 26-fold among the cell lines. SU.86.86 cells secreted the most PGK, 2.09 ± 0.10 μg per 10^6 cells in 24 hours, while AsPC-1 cells secreted the least, 0.08 ± 0.01 μg per 10^6 cells in 24 hours. In addition, a cell line cloned from Hs766T, Hs766Tf, secreted 82% more PGK in 24 hours than the parent line. There was no discernible decrease in cell viability or significant release of lactate dehydrogenase from the cells after 24 hours culture. These observations implied that secretion of PGK was a specific property of living cells and was not due to leakage from dead cells. It is noteworthy that the rate of secretion of PGK by the HT1080, breast and colon carcinoma (not shown) cells correlated with the plasmin reductase activity secreted by these cells. Moreover, the rate of secretion of PGK by the pancreatic carcinoma cells was inversely correlated with the tumourigenicity of the cells grown subcutaneously in the proximal midline dorsum of SCID mice. The tumourigenicity was in the order AsPC-1 > BxPC-3 >> SU.86.86 (not shown).

Example 7

Plasma levels of PGK and reduced angiostatin are elevated in mice with HT1080 tumors

HT1080 tumors were established subcutaneously in female 7 to 9 week old SCID mice were used (Massachusetts General Hospital, Boston, MA) (O'Reilly *et al.*, 1996) (3). Mice were held in groups of 3 to 5 at a 12 hour day and night cycle and were fed with animal chow and water ad libidum. SCID mice were anaesthetised by inhalation of isoflurane, the dorsal skin shaved and cleaned with ethanol, and a suspension of 2.5 x 10^6 HT1080 cells in 0.2 ml of DMEM was injected subcutaneously in the proximal midline. There were four control mice with no tumors and four mice with HT1080 tumors. Tumors were excised and weighed at the conclusion of the experiment. The mean tumor weight was 5.7 ± 1.0 g. The mean weight of the control and tumor bearing mice was 23 ± 3 g and 22 ± 2 g, respectively.

Blood was collected from the retroorbital plexus of the mice and one hour later were injected intraperitoneally with 10 mg per kg of rhPGK in PBS. The mice were bled again after two hours. Blood was collected into PBS containing EDTA (20 mM) to limit coagulation, aprotinin (5 μM) to inactivate plasmin, and MPB (200 μM) to label reduced angiostatin and incubated for 60 minutes at room temperature. Cysteine (400 μM) was

added to the blood after 60 minutes to quench unreacted MPB. Plasma was collected by centrifugation and assayed for PGK (see Fig. 5) or MPB-labeled angiostatin (see Fig. 1).

The plasma of mice bearing HT1080 tumours contained 6.6-fold more PGK than mice without tumours (Fig. 6a). This finding implied that relatively small amounts of PGK was secreted from normal tissues. To determine whether PGK could reduce plasmin *in vivo*, mice with and without HT1080 tumours were injected with rhPGK, it was hypothesised that the rhPGK would facilitate reduction of plasmin produced by the HT1080 tumours and result in increased plasma levels of angiostatin containing free thiols. The plasma levels of MPB-labeled angiostatin were 48% higher on average in tumour-bearing mice compared to mice without tumours (Fig. 6b). Importantly, administration of rhPGK to tumour-bearing mice resulted in 86% increase in the plasma levels of MPB-labeled angiostatin after 2 hours ($p < 0.05$). In contrast, administration of rhPGK to mice without tumours had no significant effect on plasma levels of MPB-labeled angiostatin. These findings support the proposed role for PGK in angiostatin formation and imply that blood levels of angiostatin can be increased by systemic administration of PGK.

Example 8

Administration of PGK to tumour-bearing mice resulted in decrease in tumour vascularity and rate of tumour growth

HT1080 or AsPC-1 tumors were established subcutaneously in female 7 to 9 week old SCID mice as described in Example 7. Tumours were allowed to establish and grow to a size of ~0.1 cm^3 after which they were randomised into two groups. The tumours were measured in two diameters and tumour volume was calculated using the relationship, tumour volume = $a \times b^2 \times 0.52$, where a is the longest and b the shortest diameters in cm. Animals were treated with either vehicle (PBS) or 5 mg per kg per day of rhPGK via 0.2 ml intraperitoneal injections. Tumour volume and animal weight was measured every 3 days. The tumours were excised and weighed when the animals were sacrificed.

The growth of human fibrosarcoma (HT1080) and pancreatic carcinoma (AsPC-1) tumours in immunocompromised mice was suppressed by systemic administration of rhPGK. Intraperitoneal administration of 5 mg rhPGK per kg per day caused a ~50% inhibition of the rate of HT1080 tumour growth (Fig. 7a) and ~70% inhibition of the rate of AsPC-1 tumour growth (Fig. 7b). There was no apparent adverse side effects of

administration of rhPGK to the mice and there was no macroscopic or microscopic (not shown) signs of necrosis of the rhPGK-treated tumours.

The tumours shown in Fig. 7c were fixed in Buffered Formalde-Fresh (Fisher Scientific, Fair Lawn, NJ), embedded in paraffin and five μm thick sections were cut and placed on glass slides. Sections were stained with haematoxylin and eosin or for CD31. Sections were incubated overnight at 4°C with a 1:250 dilution of anti-mouse CD-31 antibody (PharMingen, San Diego, CA) followed by a 1:200 dilution of biotinylated anti-rat secondary antibody (Vestor, Burlingame, CA). The staining was enhanced by tyramide amplification (New England Nuclear, Boston, MA). Vascular density was determined by selecting 3 tumours, including the smallest and largest, from the control and treatment groups. Microvessels were counted and their density was graded in the most active areas of neovascularisation (9). Sections were examined under 100× magnification to find the areas of most active neovascularisation and three different fields were counted at 400× magnification for the number of microvessels. The highest of the three counts was the value taken and two sections from each tumour was examined.

Immunohistochemical staining of the AsPC-1 tumours for vascularity indicated a marked reduction in angiogenesis in the rhPGK-treated tumours (Fig. 7d). There was 80 ± 13 vessels per field in the rhPGK-treated tumours compared to 173 ± 9 vessels per field in the vehicle-treated tumours ($p < 0.05$). It is interesting that AsPC-1 tumourigenesis was more susceptible to suppression by systemic administration of PGK than HT1080 tumourigenesis. This may reflect the 5-fold reduced secretion of PGK by AsPC-1 cells compared to HT1080 cells (Fig. 5).

Example 9

PGK is elevated in the plasma of cancer patients

Sheep polyclonal antibodies were developed against purified hrPGK and affinity purified on a rhPGK-AffiGel15 matrix (Biorad Laboratories, Hercules, CA). Horseradish peroxidase was coupled to the affinity-purified antibodies using the EZ-Link Activated Peroxidase and Antibody Labeling Kit from Pierce, Rockford, IL. Affinity purified anti-PGK polyclonal antibodies (100 μl of 5 μg per ml in 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% azide, pH 9.6 buffer) were adsorbed to Nunc PolySorp 96 well plates overnight at 4°C in a humid environment. Wells were washed once with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS/Tween), non-specific binding sites blocked by adding 200 μl of 2% BSA in PBS and incubating for 90 minutes at 37°C, and then washed

two times with PBS/Tween. Normal or patient plasmas were diluted 1:5 in PBS/Tween and 100 µl aliquots added to antibody coated wells and incubated for 30 minutes at room temperature with orbital shaking. The positive controls were normal plasma spiked with 0.1, 0.5 or 1 µg per ml rhPGK. Wells were washed three times with PBS/Tween and 100 µl of 1 µg per ml of the peroxidase-conjugated anti-PGK polyclonal antibodies added and incubated for 30 minutes at room temperature with orbital shaking. Wells were washed three times with PBS/Tween and the colour developed with 100 µl of 0.003% H_2O_2, 1 mg/ml 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in 50 mM citrate, pH 4.5 buffer for 20 minutes at room temperature with orbital shaking. Absorbances were read at 405 nm using a Molecular Devices Thermomax Kinetic Microplate Reader (Molecular Devices Corporation, CA, USA). Results were corrected for control wells not coated with polyclonal antibody and incubated with normal plasma.

The plasma levels of PGK in 8 patients with non-identified metastatic breast or colon cancer were all higher than the PGK level of pooled normal plasma. The plasma levels of PGK in two patients in particular (#6100 and #6156) were at least an order of magnitude higher than the PGK level in healthy subject plasma.

Example 10

Phosphoglycerate kinase (PGK) may be administered alone, although it is preferable that it be administered as a pharmaceutical formulation. The active ingredient may comprise, for topical administration, from 0.001% to 10% by weight, eg., from 1% to 5% by weight of the formulation, although it may comprise as much as 10% by weight but preferably not in excess of 5% by weight, and more preferably from 0.1% to 1% by weight of the formulation.

In accordance with the description of the invention provided above specific preferred pharmaceutical compositions of the present invention may be prepared, and examples of which are provided below. The following specific formulations are to be construed as merely illustrative examples of formulations and not as a limitation of the scope of the present invention in any way.

Example 10(a) - Topical Cream Composition

A typical composition for delivery as a topical cream is outlined below:

PGK	1.0 g
Polawax GP 200	30.0 g
Lanolin Anhydrous	3.0 g

White Beeswax	5.5 g
Methyl hydroxybenzoate	0.2 g
Sterilised isotonic saline to	100.0 g

The polawax, beeswax and lanolin are heated together at 60°C, a solution of methyl hydroxybenzoate is added and homogenisation is achieved using high speed stirring. The temperature is then allowed to fall to 50°C. PGK is then added and dispersed throughout, and the composition is allowed to cool with slow speed stirring.

Example 10(b) - Topical Lotion Composition

A typical composition for delivery as a topical lotion is outlined below:

PGK	1.2 g
Sorbitan Monolaurate	0.65 g
Polysorbate 20	0.75 g
Cetostearyl Alcohol	1.8 g
Glycerin	9.0 g
Methyl Hydroxybenzoate	0.25 g
Isotonic saline to	100.00 ml

The methyl hydroxybenzoate and glycerin are dissolved in 70 ml of the isotonic saline at 75°C. The sorbitan monolaurate, polysorbate 20 and cetostearyl alcohol are melted together at 75°C and added to the aqueous solution. The resulting emulsion is homogenised, allowed to cool with continuous stirring and PGK is added as a suspension in the remaining water. The whole suspension is stirred until homogenised.

Example 10(c) - Eye Drop Composition

A typical composition for delivery as an eye drop is outlined below:

PGK	0.3 g
Methyl Hydroxybenzoate	0.003 g
Propyl Hydroxybenzoate	0.08 g
Purified isotonic saline to about	100.00 ml.

The methyl and propyl hydroxybenzoates are dissolved in 70 ml isotonic saline at 75°C, and the resulting solution is allowed to cool. PGK is then added, and the solution sterilised by filtration through a membrane filter (0.22 μm pore size), and aseptically packed into sterile containers.

Example 10(d) - Composition for Inhalation Administration

For an aerosol container with a capacity of 20-30 ml: a mixture of 10 mg of PGK with 0.5-1.0% by weight of a lubricating agent, such as polysorbate 85 or oleic acid, and mixture was dispersed in a propellant, such as freon, and put into an appropriate aerosol container for either intranasal or oral inhalation administration.

Example 10(e) - Composition for Parenteral Administration

A pharmaceutical composition of the present invention for intramuscular injection could be prepared to contain 1 mL sterile isotonic saline, and 1 mg of PGK.

Similarly, a pharmaceutical composition for intravenous infusion may comprise 250 ml of sterile Ringer's solution, and 5 mg of PGK.

Example 10(f) - Capsule Composition

A pharmaceutical composition PGK in the form of a capsule may be prepared by filling a standard two-piece hard gelatin capsule with 50 mg of PGK, in powdered form, 80 mg of lactose, 45 mg of talc and 12 mg of magnesium stearate.

Example 10(g) - Injectable Parenteral Composition

A pharmaceutical composition of this invention in a form suitable for administration by injection may be prepared by mixing 1% by weight of PGK in 12% by volume propylene glycol and isotonic saline. The solution is sterilised by filtration.

Example 10(h) - Ointment Composition

A typical composition for delivery as an ointment includes 1.5g of PGK, together with white soft paraffin to 100.0 g, is dispersed to produce a smooth, homogeneous product.

Example 11

PGK may also be administered with a plasminogen activator, either alone, or more preferably as a pharmaceutical formulation. The following specific formulations are to be construed as merely illustrative examples of formulations and not as a limitation of the scope of the present invention in any way.

Example 11(a) - Topical Cream Composition

A typical composition for delivery as a topical cream is outlined below:

PGK	1.0 g
Streptokinase	0.8g
Polawax GP 200	28.0 g

Lanolin Anhydrous	2.50 g
White Beeswax	4.8 g
Methyl hydroxybenzoate	0.2 g
Isotonic saline to	100.0 g

The polawax, beeswax and lanolin are heated together at 60°C, a solution of methyl hydroxybenzoate is added and homogenisation is achieved using high speed stirring. The temperature is then allowed to fall to 50°C. PGK and streptokinase is then added and dispersed throughout, and the composition is allowed to cool with slow speed stirring.

Example 11(b) - Topical Lotion Composition

A typical composition for delivery as a topical lotion is outlined below:

PGK	1.2 g
Streptokinase	0.8g
Sorbitan Monolaurate	1.0 g
Polysorbate 20	0.7 g
Cetostearyl Alcohol	2.0 g
Glycerin	7.5 g
Methyl Hydroxybenzoate	0.5 g
Sterilised isotonic saline to about	100.00 ml

The methyl hydroxybenzoate and glycerin are dissolved in 70 ml of the isotonic saline at 75°C. The sorbitan monolaurate, polysorbate 20 and cetostearyl alcohol are melted together at 75°C and added to the aqueous solution. The resulting emulsion is homogenised, allowed to cool with continuous stirring and PGK and streptokinase are added as a suspension in the remaining water. The whole suspension is stirred until homogenised.

Example 11(c) - Eye Drop Composition

A typical composition for delivery as an eye drop is outlined below:

PGK	0.3 g
Streptokinase	0.8g
Methyl Hydroxybenzoate	0.005 g
Propyl Hydroxybenzoate	0.06 g
Purified isotonic saline to about	100.00 ml.

The methyl and propyl hydroxybenzoates are dissolved in 70 ml isotonic saline at 75°C, and the resulting solution is allowed to cool. PGK and streptokinase are then added, and the solution sterilised by filtration through a membrane filter (0.22 μm pore size), and aseptically packed into sterile containers.

Example 11(d) - Composition for Inhalation Administration

For an aerosol container with a capacity of 20-30 ml: a mixture of 10 mg of PGK, 8mg of streptokinase, together with 0.5-0.8% by weight of a lubricating agent, such as polysorbate 85 or oleic acid, and mixture was dispersed in a propellant, such as freon, and put into an appropriate aerosol container for either intranasal or oral inhalation administration.

Example 11(e) - Composition for Parenteral Administration

A pharmaceutical composition of the present invention for intramuscular injection could be prepared to contain 1 mL isotonic saline, 1 mg of PGK and 0.6 mg of streptokinase.

Similarly, a pharmaceutical composition for intravenous infusion may comprise 250 ml of sterile Ringer's solution, 5 mg of PGK and 2 mg streptokinase.

Example 11(f) - Capsule Composition

A pharmaceutical composition PGK in the form of a capsule may be prepared by filling a standard two-piece hard gelatin capsule with 50 mg of PGK and 35 mg streptokinase, in powdered form, 100 mg of lactose, 35 mg of talc and 10 mg of magnesium stearate.

Example 11(g) - Injectable Parenteral Composition

A pharmaceutical composition of this invention in a form suitable for administration by injection may be prepared by mixing 1% by weight of PGK and 0.75% streptokinase in 10% by volume propylene glycol and water. The solution is sterilised by filtration.

Example 11(h) - Ointment Composition

A typical composition for delivery as an ointment includes 1.0g of PGK and 0.75g streptokinase, together with white soft paraffin to 100.0 g, is dispersed to produce a smooth, homogeneous product.

Industrial Applicability

The present invention provides a pharmaceutical composition for the inhibition of angiogenesis associated with disease in a vertebrate, said composition comprising phosphoglycerate kinase, or a fragment(s) or analogue thereof, together with a pharmaceutically acceptable carrier, adjuvant and/or diluent. The present invention also relates to the treatment of disease associated with angiogenesis, especially cancer, in a vertebrate via the administration of a therapeutically effective amount of phosphoglycerate kinase (PGK), or a fragment(s) or analogue thereof.

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CLAIMS

1. A pharmaceutical composition for the inhibition of angiogenesis associated with disease in a vertebrate, said composition comprising phosphoglycerate kinase, or a fragment(s) or analogue thereof, together with a pharmaceutically acceptable carrier, adjuvant and/or diluent.

2. The pharmaceutical composition of claim 1, wherein the phosphoglycerate kinase (PGK) is ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3.

3. The pharmaceutical composition of claim 1, wherein the phosphoglycerate kinase is encoded by the isolated nucleic acid molecule of SEQ ID NO: 1,or an analogue thereof.

4. The pharmaceutical composition of claim 3, wherein the analogue has at least 45% homology to the polynucleotide of SEQ ID NO:1.

5. The pharmaceutical composition of claim 3, wherein the analogue has at least 75% homology to the polynucleotide of SEQ ID NO:1.

6. The pharmaceutical composition of claim 3, wherein the analogue has at least 90% homology to the polynucleotide of SEQ ID NO:1.

7. The pharmaceutical composition of any one of claims 1-6, wherein the nucleic acid molecule of SEQ ID NO:1 is an oligonucleotide fragment thereof.

8. The pharmaceutical composition of claim 7, wherein said oligonucleotide fragment is between about 15 to about 750 nucleotides in length.

9. The pharmaceutical composition of claim 7, wherein said oligonucleotide fragment is between about 15 to about 150 nucleotides in length.

10. The pharmaceutical composition of claim 7, wherein said oligonucleotide fragment is between about 15 to about 75 nucleotides in length.

11. The pharmaceutical composition of claim 1, wherein the phosphoglycerate kinase comprises the amino acid sequence of SEQ ID NO: 2, or a fragment(s) or analogue thereof.

12. The pharmaceutical composition of claim 11, wherein the fragment comprises between about 5 to about 250 contiguous amino acids.

13. The pharmaceutical composition of claim 11, wherein the fragment comprises between about 5 to about 100 contiguous amino acids.

14. The pharmaceutical composition of claim 11, wherein the fragment comprises between about 5 to about 20 contiguous amino acids.

15. The pharmaceutical composition of any one of claims 1-14, wherein PGK is present in a form selected from the group consisting of: PGK/chelate, PGK/drug, PGK/prodrug, PGK/toxin and PGK/detector group and PGK/imaging marker.

16. The pharmaceutical composition of claim 15, wherein the chelate is selected from the group consisting of: gadolinium, ^{90}Y, ^{131}I and ^{188}Re.

17. The pharmaceutical composition of claim 15, wherein the drug is a cytotoxic drug.

18. The pharmaceutical composition of claim 17, wherein the cytotoxic drug is may be selected from the group consisting of: adriamycin, melphalan, cisplatin, taxol, fluorouricil and cyclophosphamide.

19. The pharmaceutical composition of claim 15, wherein the toxin is selected from the group consisting of: ricin, abrin, *Diptheria* toxin and *Pseudomonas* endotoxin (PE 40).

20. The pharmaceutical composition of claim 15, wherein the detector group is selected from the group consisting of: biotin, streptavidin, dioxigenin, ^{3}H, ^{14}C, ^{32}P, ^{33}P, ^{35}S, ^{125}I, ^{131}I, ^{123}I, ^{111}In, ^{105}Rh, ^{153}Sm, ^{67}Cu, ^{67}Ga, ^{166}Ho, ^{177}Lu, ^{186}Re, ^{188}Re, and ^{99m}Tc.

21. The pharmaceutical composition of any one of claims 1-20, wherein the vertebrate is selected from the group consisting of human, non-human primate, murine, bovine, ovine, equine, caprine, leporine, avian, feline and canine.

22. The pharmaceutical composition of claim 21, wherein the vertebrate is human.

23. A process for preparing the pharmaceutical composition of any one of claims 1-22, wherein said process comprises homogeneously mixing a phosphoglycerate kinase, or a fragment(s) or analogue thereof, with a pharmaceutically acceptable carrier, adjuvant and/or diluent.

24. A method for the treatment of disease in a vertebrate in need of said treatment, wherein said method comprises administering to said vertebrate, a therapeutically effective amount of phosphoglycerate kinase, or a fragment(s) or analogue thereof.

25. A method for the treatment of disease in a vertebrate in need of said treatment, wherein said method comprises administering to said vertebrate, a

therapeutically effective amount of the pharmaceutical composition of any one of claims 1-22.

26. The method of claim 24 or 25, wherein the vertebrate is selected from the group consisting of human, non-human primate, murine, bovine, ovine, equine, caprine, leporine, avian, feline and canine.

27. The method of claim 26, wherein the vertebrate is human.

28. A pharmaceutical composition for inhibition of angiogenesis associated with disease in a vertebrate, wherein said composition comprises phosphoglycerate kinase, or a fragment(s) or analogue thereof, and at least one plasminogen activator.

29. The pharmaceutical composition of claim 28, wherein said plasminogen activator is selected from the group consisting of: streptokinase, tissue plasminogen activator, and staphylokinase or urokinase plasminogen activator.

30. The pharmaceutical composition of claim 28 or 29, wherein said composition further comprises a pharmaceutically acceptable carrier, adjuvant and/or diluent.

31. The pharmaceutical composition of any one of claims 28-30, wherein the vertebrate is selected from the group consisting of human, non-human primate, murine, bovine, ovine, equine, caprine, leporine, avian, feline and canine.

32. The pharmaceutical composition of claim 31, wherein the vertebrate is human.

33. A method for the treatment of disease in a vertebrate in need of said treatment, wherein said method comprises administering to said vertebrate a therapeutically effective amount of the composition of any one of claims 28-32.

34. A method for the treatment of disease in a vertebrate in need of said treatment, wherein said method comprises simultaneously administering to said vertebrate, a therapeutically effective amount of phosphoglycerate kinase, or a fragment(s) or analogue thereof, and at least one plasminogen activator.

35. The method of any one of claims 24-27 or 33-34, wherein said method is used in conjunction with other disease treatments.

36. The method of claim 35, wherein other disease treatments include surgery, radiation treatment, or chemotherapy.

37. A method for screening for a disease in a vertebrate comprising:

(a) contacting a sample from a vertebrate with an antibody (or fragment thereof) raised against a PGK polypeptide (or fragment thereof), and

(b) detecting the presence of the antibody (or fragment thereof) bound to the PGK polypeptide.

38. The method of claim 37, wherein said sample is a plasma sample.

39. The method of any one of claims 24-27 or 33-38, wherein said disease is an angiogenesis-dependent disease.

40. The method of claim 39, wherein said angiogenesis-dependent disease is selected from the group consisting of: cancer, solid tumours, hemangioma, arteriovenous malformations, arthritis, Osler-Weber Syndrome, atherosclerotic plaques, psoriasis, corneal graft neovascularization, pyrogenic granuloma, wound healing, retrolental fibroplasia, diabetic retinopathy, scleroderma, granulations, angiofibroma, neovascular glaucoma, trachoma, hemophilic joints, hypertrophic scars, or gastric ulcers.

41. The method of claim 40, wherein said cancer is a haematological tumour.

42. The method of claim 40, wherein said cancer is a solid tumour.

43. The method of claim 42, wherein said solid tumours are selected from the group consisting of: solid tumours arising from hematopoietic malignancies, chloromas, plasmacytomas, plaques, tumours of mycosis fungoides and cutaneous T-cell lymphoma/leukaemia; and lymphomas.

44. The method of claim 43, wherein said lymphoma is Hodgkin's or non-Hodgkin's lymphoma.

45. The method of claim 40, wherein said cancer is selected from the group consisting of cancers of: oropharynx; hypopharynx; oesophagus; stomach; pancreas; liver; gallbladder; bile ducts; small intestine; urinary tract including kidney, bladder and urothelium; female genital tract including cervix, uterus, ovaries, choriocarcinoma and gestational trophoblastic disease; male genital tract including prostate, seminal vesicles, testes and germ cell tumours; endocrine glands including thyroid, adrenal, and pituitary; skin including hemangiomas, melanomas, sarcomas arising from bone or soft tissues and Kaposi's sarcoma; tumours of the brain, nerves, eyes, and meninges including astrocytomas, gliomas, glioblastomas, retinoblastomas, neuromas, neuroblastomas, Schwannomas and meningiomas.

46. A method for increasing circulatory phosphoglycerate kinase levels in a vertebrate in need of said increase, wherein said method comprises generating anti anti-PGK antibodies and/or fragments thereof; and administering a therapeutically effective amount of said anti anti-PGK antibodies and/or fragments thereof to said vertebrate.

47. The method of claim 46, wherein the administration of anti anti-PGK antibodies and/or fragments thereof reduces the removal of circulating PGK by anti-PGK antibodies.

48. A method for inhibiting the action of excess endogenous phosphoglycerate kinase in a vertebrate in need of said inhibition, wherein said method comprises passively immunising said vertebrate with anti-PGK antibodies and/or fragments thereof.

49. A diagnostic kit for the detection of disease in a vertebrate, said kit comprising at least an antibody (or fragment thereof) raised against PGK (or fragment thereof), together with a diagnostically acceptable carrier and/or diluent.

50. The kit of claim 49, wherein said kit comprises:

(a) a first container containing at least the antibody (or fragment thereof) raised against PGK (or fragment thereof), and;

(b) a second container containing a conjugate comprising a binding partner of the antibody (or fragment thereof), together with a detectable label.

51. A method of gene therapy for the inhibition of angiogenesis associated with disease in a vertebrate, wherein said method comprises:

(a) inserting a nucleic acid molecule encoding for a phosphoglycerate kinase, or a fragment(s) or analogue thereof, or a vector comprising a nucleic acid molecule encoding for a phosphoglycerate kinase, or a fragment(s) or analogue thereof, into a host cell;

(b) expressing the nucleic acid molecule in the transformed cell.

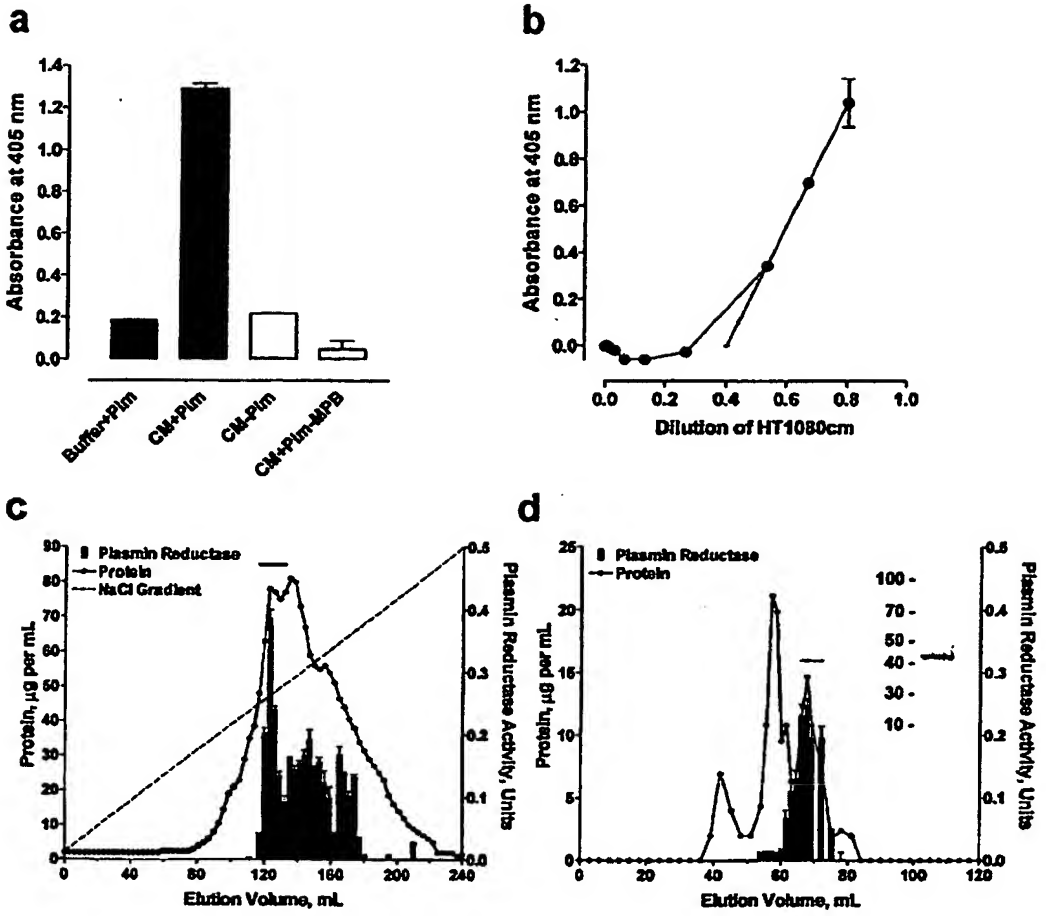
a
Absorbance at 405 nm
Buffer+Plm
CM+Plm
CM-Plm
CM+Plm-MPB
b
Absorbance at 405 nm
Dilution of HT1080cm
c
Plasmin Reductase
Protein
NaCl Gradient
Protein, µg per mL
Plasmin Reductase Activity, Units
Elution Volume, mL
d
Plasmin Reductase
Protein
Protein, µg per mL
Plasmin Reductase Activity, Units
Elution Volume, mL

Figure 1

```
1   MSLSNKLTLD KLDVKGKRVV MRVDFNVPMK NNQITNNQRI KAAVPSIKFC
                           RVDFN
51  LDNGAKSVVL MSHLGRPDGV PMPDKYSLEP VAVELKSLLG KDVLFLKDCV
              SHLGRPDG
101 GPEVEKACAN PAAGSVILLE NLRFHVEEEG KGKDASGNKV KAEPAKIEAF

151 RASLSKLGDV YVNDAFGTAH RAHSSMVGVN LPQKAGGFLM KKELNYFAKA
                               VGVN LPQKAGG
201 LESPERPFLA ILGGAKVADK IQLINNMLDK VNEMIIGGGM AFTFLKVLNN
                                                AFTFLKVLNN
251 MEIGTSLFDE EGAKIVKDLM SKAEKNGVKI TLPVDFVTAD KFDENAKTGQ

301 ATVASGIPAG WMGLDCGPES SKKYAEAVTR AKQIVWNGPV GVFEWEAFAR

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401 ELLEGKVLPG VDALSNIL
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Figure 2

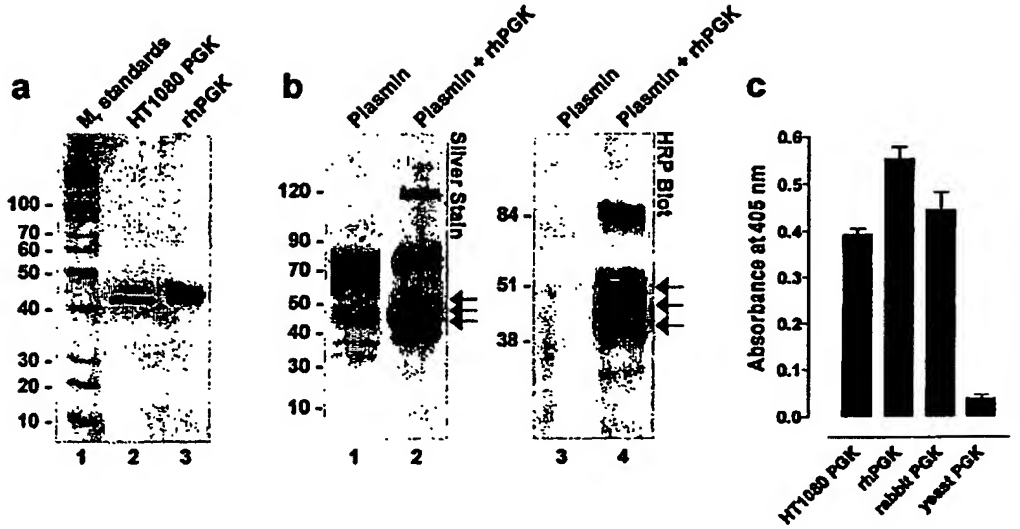
a
M, standards
HT1080 PGK
rhPGK
100
70
60
50
40
30
20
10
1
2
3
b
Plasmin
Plasmin + rhPGK
Silver Stain
120
90
70
50
40
30
10
1
2
Plasmin
Plasmin + rhPGK
HRP Blot
84
51
38
3
4
c
Absorbance at 405 nm
0.6
0.5
0.4
0.3
0.2
0.1
0.0
HT1080 PGK
rhPGK
rabbit PGK
yeast PGK

Figure 3

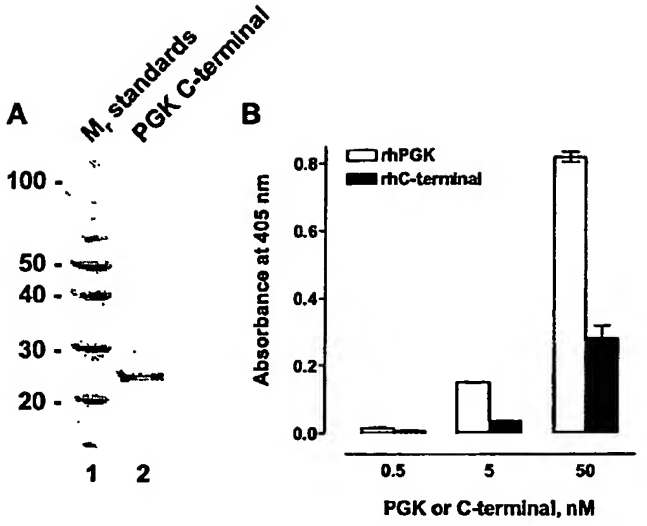
A
M, standards
PGK C-terminal
100 -
50 -
40 -
30 -
20 -
1
2
B
rhPGK
rhC-terminal
Absorbance at 405 nm
0.8
0.6
0.4
0.2
0.0
0.5
5
50
PGK or C-terminal, nM

Figure 4

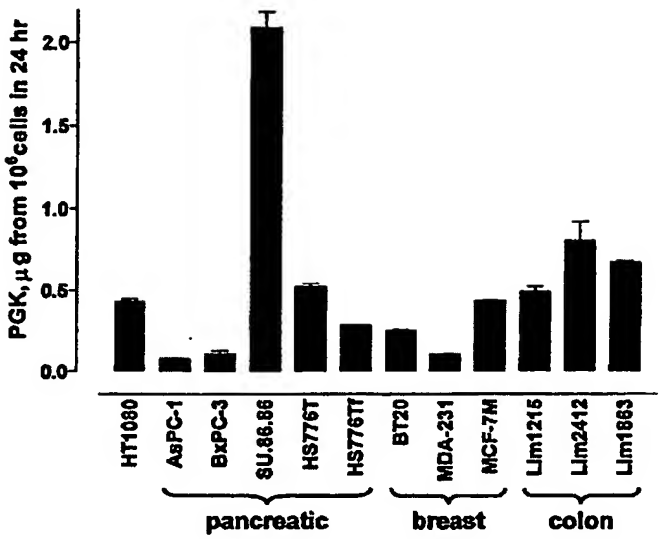
PGK, µg from 10^6 cells in 24 hr
2.0
1.5
1.0
0.5
0.0
HT1080
AsPC-1
BxPC-3
SU.86.86
HS776T
HS776T
BT20
MDA-231
MCF-7M
Lim1215
Lim2412
Lim1863
pancreatic
breast
colon

Figure 5

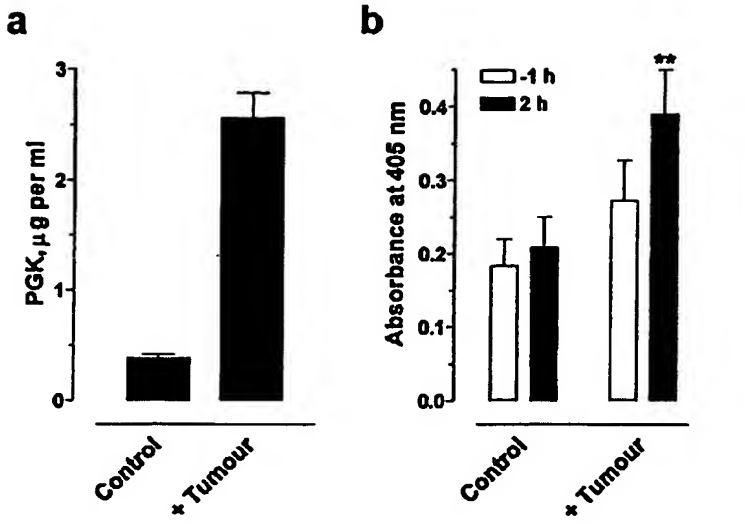
a
b
PGK, µg per ml
3
2
1
0
Control
* Tumour
Absorbance at 405 nm
0.4
0.3
0.2
0.1
0.0
-1 h
2 h
**
Control
* Tumour

Figure 6

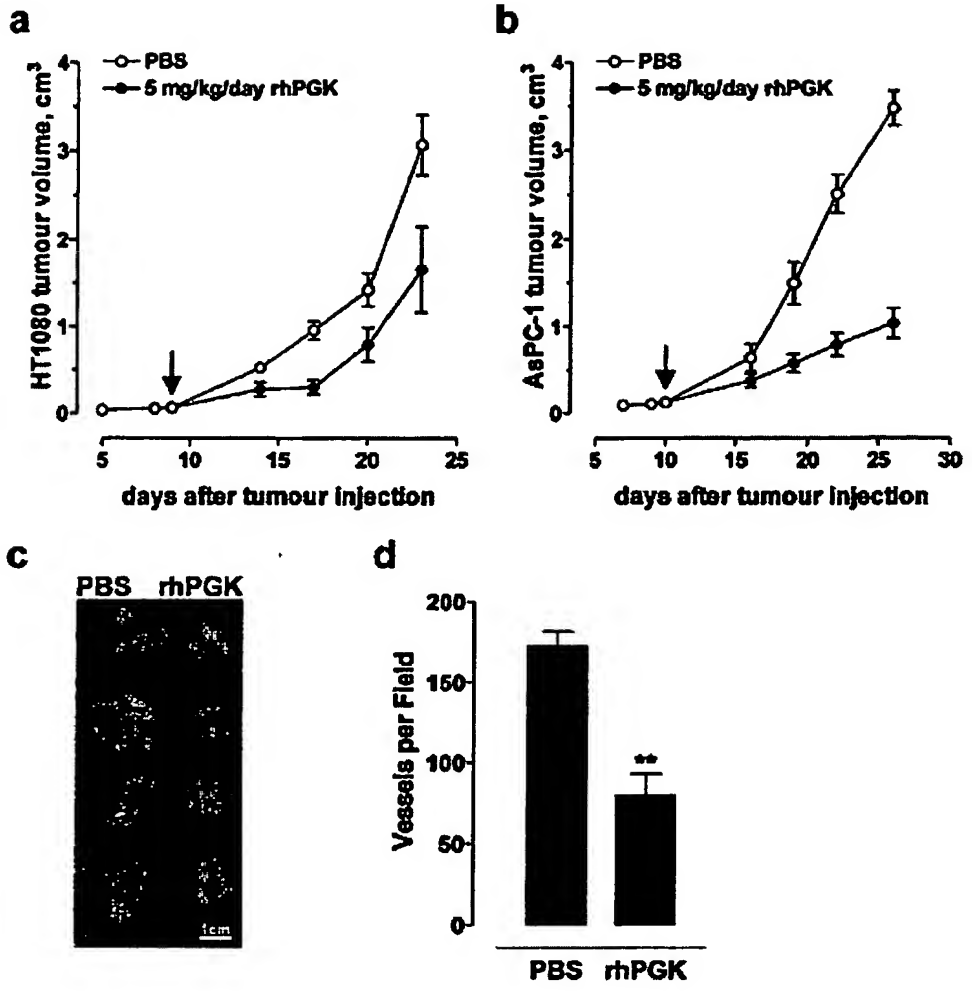
a
PBS
5 mg/kg/day rhPGK
HT1080 tumour volume, cm³
days after tumour injection
b
PBS
5 mg/kg/day rhPGK
AsPC-1 tumour volume, cm³
days after tumour injection
c
PBS rhPGK
1cm
d
Vessels per Field
**
PBS rhPGK

Figure 7

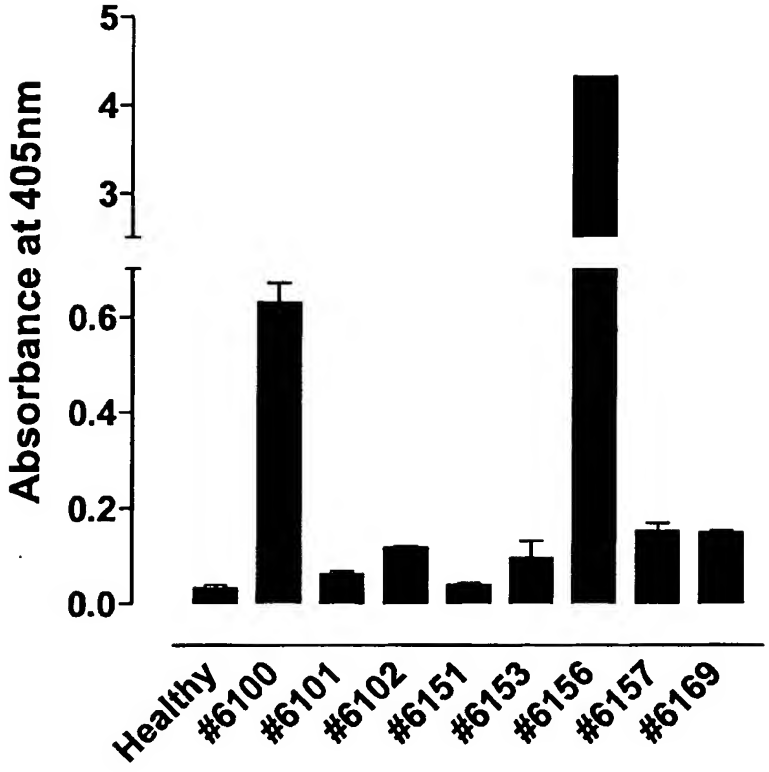
Absorbance at 405nm
5
4
3
0.6
0.4
0.2
0.0
Healthy
#6100
#6101
#6102
#6151
#6153
#6156
#6157
#6169

Figure 8

Sequ nce Listing

<110> Unisearch Limited; Angelina Jay Lay; Philip John Hogg

<120> Phosphoglycerate Kinase in the Treatment of Disease

<130> 487200C

<150> PQ4754

<151> 17 December 1999

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181   AACAAACAAC CAGAGGATTA AGGCTGCTGT CCCAAGCATC AAATTCTGCT TGGACAATGG
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301   CAAGTACTCC TTAGAGCCAG TTGCTGTAGA ACTCAAATCT CTGCTGGGCA AGGATGTTCT
361   GTTCTTGAAG GACTGTGTAG GCCCAGAAGT GGAGAAAGCC TGTGCCAACC CAGCTGCTGG
421   GTCTGTCATC CTGCTGGAGA ACCTCCGCTT TCATGTGGAG GAAGAAGGGA AGGGAAAAGA
481   TGCTTCTGGG AACAAGGTTA AAGCCGAGCC AGCCAAAATA GAAGCTTTCC GAGCTTCACT
541   TTCCAAGCTA GGGGATGTCT ATGTCAATGA TGCTTTTGGC ACTGCTCACA GAGCCCACAG
601   CTCCATGGTA GGAGTCAATC TGCCACAGAA GGCTGGTGGG TTTTTGATGA AGAAGGAGCT
661   GAACTACTTT GCAAAGGCCT TGGAGAGCCC AGAGCGACCC TTCCTGGCCA TCCTGGGCGG
721   AGCTAAAGTT GCAGACAAGA TCCAGCTCAT CAATAATATG CTGGACAAAG TCAATGAGAT
781   GATTATTGGT GGTGGAATGG CTTTTACCTT CCTTAAGGTG CTCAACAACA TGGAGATTGG
841   CACTTCTCTG TTTGATGAAG AGGGAGCCAA GATTGTCAAA GACCTAATGT CCAAAGCTGA
901   GAAGAATGGT GTGAAGATTA CCTTGCCTGT TGACTTTGTC ACTGCTGACA AGTTTGATGA
961   GAATGCCAAG ACTGGCCAAG CCACTGTGGC TTCTGGCATA CCTGCTGGCT GGATGGGCTT
1021  GGACTGTGGT CCTGAAAGCA GCAAGAAGTA TGCTGAGGCT GTCACTCGGG CTAAGCAGAT
1081  TGTGTGGAAT GGTCCTGTGG GGGTATTTGA ATGGGAAGCT TTTGCCCGGG GAACCAAAGC
1141  TCTCATGGAT GAGGTGGTGA AAGCCACTTC TAGGGGCTGC ATCACCATCA TAGGTGGTGG
1201  AGACACTGCC ACTTGCTGTG CCAAATGGAA CACGGAGGAT AAAGTCAGCC ATGTGAGCAC
1261  TGGGGGTGGT GCCAGTTTGG AGCTCCTGGA AGGTAAAGTC CTTCCTGGGG TGGATGCTCT
1321  CAGCAATATT TAGTACTTTC CTGCCTTTTA GTTCCTGTGC ACAGCCCCTA AGTCAACTTA
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1381 GCATTTTCTG CATCTCCACT TGGCATTAGC TAAAACCTTC CATGTCAAGA TTCAGCTAGT
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1501 ACTGCACCCT GGATTTGCAT ACATTCTTCA AGATCCCATT TGAATTTTTT AGTGACTAAA
1561 CCATTGTGCA TTCTAGAGTG CATATATTTA TATTTTGCCT GTTAAAAAGA AAGTGAGCAG
1621 TGTTAGCTTA GTTCTCTTTT GATGTAGGTT ATTATGATTA GCTTTGTCAC TGTTTCACTA
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1741 TTAAGTAAAC AATAAAAGTG TCCATTG

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<150> PQ4754
<151> 17 December 1999
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151 RASLSKLGDV YVNDAFGTAH RAHSSMVGVN LPQKAGGFLM KKELNYFAKA
201 LESPERPFLA ILGGAKVADK IQLINNMLDK VNEMIIGGGM AFTFLKVLNN
251 MEIGTSLFDE EGAKIVKDLM SKAEKNGVKI TLPVDFVTAD KFDENAKTGQ
301 ATVASGIPAG WMGLDCGPES SKKYAEAVTR AKQIVWNGPV GVFEWEAFAR
351 GTKALMDEVV KATSRGCITI IGGGDTATCC AKWNTEDKVS HVSTGGGASL
401 ELLEGKVLPG VDALSNI

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/01542

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: A61K 38/44, 39/395, 48/00; A61P 35/00; G01N 33/574, 33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IP7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE DATABASES BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, Chemical Abstracts, Medline: Keywords used - phosphoglycerate kinase, pgk, plasmin reductase, angiogenesis, angiostatin, cancer, tumour, tumor, metastasis, neoplasm, carcinoma
Swiss-Prot, Gen-Pept, PIR, TREMBL, GenBank, EMBL - SEQ. ID. NOs: 1, 2

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	LAY A.J. et al. "Phosphoglycerate kinase acts in tumour angiogenesis as a disulphide reductase" Nature (2000, December) volume 408, pages 869-873. See entire document	1-56
A	STATHAKIS P. et al. "Angiostatin formation involves disulphide bond reduction and proteolysis in kringle 5 of plasmin" The Journal Of Biological Chemistry (1999, March) volume 274, number 13, pages 8910-8916. See entire document.	1-56

[X] Further documents are listed in the continuation of Box C [ ] See patent family annex

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search
31 January 2001

Date of mailing of the international search report
8 February 2001

Name and mailing address of the ISA/AU

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/01542

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HANAHAN D. and FOLKMAN J. "Patterns and emerging mechanisms of the angiogenic switch during tumjorigenesis" Cell (1996) volume 86, pages 353-364. See entire document.	1-56
A	MICHELSON A.M. et al. "Isolation and DNA sequence of full-length cDNA clone for human X chromosome-encoded phosphoglycerate kinase" Proceedings Of The National Academy Of Sciences USA (1983) volume 80, pages 472-476. See entire document.	1-56